ir. Bob ASSELBERGH

THE ROLE OF ABSCISIC ACID IN THE DEFENCE RESPONSE OF TOMATO (*SOLANUM LYCOPERSICUM*) TO THE NECROTROPHIC PATHOGENS *BOTRYTIS CINEREA* AND *ERWINIA CHRYSANTHEMI*

Thesis submitted in fulfillment of the requirements for the degree of Doctor (PhD) in Applied Biological Sciences – Cell and Gene Biotechnology

Wat was wetenschap voor hem? Het willen weten van dingen waar men nieuwschierig naar zijn moest om er belang in te stellen.

Een mier dwalend door een spons, was hij. Nooit zou hij in alle gangen kunnen komen, daartoe was de tijd te kort.

Willem Frederik Hermans, *De tranen der acacia's*, 1971.

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Titel van het doctoraat in het Nederlands

De rol van abscisinezuur in de afweer van tomaat (*Solanum lycopersicum*) tegen de necrotrofe pathogenen *Botrytis cinerea* en *Erwinia chrysanthemi*

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LIST OF ABBREVIATIONS

2-DDG	2-deoxy-D-glucose
ABA	abscisic acid
ABRE	ABA-responsive elements
AGPs	arabinogalactan proteins
AM	arbuscular mycorrhizal
ANOVA	analysis of variance
BABA	β-amino butyric acid
BTH	benzothiadiazole
cDNA	complement DNA (deoxyribonucleic acid)
CF	culture filtrate
CFU	colony-forming units
CWDEs	cell wall-degrading enzymes
DAB	3,3'-diaminobenzidine
ERF	ethylene response factor
ESTs	expressed sequence tags
ET	ethylene
DPI	diphenilene iodonium
dpi	days post inoculation
FC	fold change
FITC	fluorescein isothiocyanate
GPI	glycosylphosphatidylinositol
H_2O_2	hydrogen peroxide
hpi	hours post inoculation
HR	hypersensitive response
Hrp	hypersensitive response and pathogenicity
JA	jasmonic acid
LRW	LR White resin
MAPK	mitogen-activated protein kinase
nahG	salicylate hydroxylase
NBT	nitro blue tetrazolium
NCED	nine-cis epoxycaretenoid dioxygenase

OGA	oligogalacturonide
PAL	phenylalanine ammonia lyase
PAMPs	pathogen-associated molecular patterns
PCD	programmed cell death
PD	degree of polymerisation
PGA	polygalacturonic acid
PME	pectin methylesterase
PR	pathogenesis-related
REML	residual maximum likelihood
R-gene	resistance gene
RH	relative humidity
ROS	reactive oxygen species
T3SE	type III secreted effectors
TMB	tetramethylbenzoidine
SA	salicylic acid
SAR	systemic acquired resistance
SNARE	specific N-ethyl-malmeimide-sensitive fusion protein attachment protein receptors

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Thesis Outline

Modern agriculture is facing important constraints. Restriction of chemical protection treatments in integrated crop management makes farmers more dependable on alternative disease control measures. An important function is assigned to crop protection methods that are based on natural plant defence mechanisms. A second challenge that plant growers are facing worldwide are the huge economical losses caused by salt, temperature and drought stress. The incidence and effects of these stresses are believed to increase further in the future as a result of climate evolution. Therefore, plant breeders give high priority to the improvement of abiotic stress tolerance and biotic stress resistance. Fundamental knowledge on how plants cope with the regulation and coordination of responses to different stresses is essential to engage in these challenges. The plant hormone abscisic acid (ABA) has a well studied role in response to abiotic stress, such as cold, drought and salt stresses, but its role in response to pathogens has long been a neglected field of research. In this thesis we aim to expand our knowledge on the functions of ABA in plant defence to pathogens.

Knowledge on the role of ABA in plant-pathogen interactions is puny, especially compared to the vast amounts of studies dealing with plant hormonal regulation of disease signalling on the one hand, and the well-established function of ABA in response to abiotic stresses on the other hand. In most plant-pathogen interactions studied so far, a negative role on disease resistance is given to ABA: high ABA levels are generally associated with susceptibility, while ABA reduction often causes resistance (reviewed by Mauch-Mani and Mauch, 2005). In our lab, the effect of ABA levels on tomato defence responses was studied by use of ABA-deficient tomato mutants. It was shown in our lab that the ABA-deficient tomato mutant *sitiens* is more resistant to the fungal necrotrophic pathogens *Botrytis cinerea* (Audenaert et al., 2002a) and *Sclerotinia sclerotiorum* (Rotthier, 2004) and to the fungal biotrophic pathogen *Oidium neolycopersici* (Achuo et al., 2006). In the present work we demonstrate that *sitiens* also displays a very high level of resistance to the necrotrophic bacterial pathogen *Erwinia chrysanthemi* (see chapter 5).

Plant defence against necrotrophic pathogens is believed to differ from defence against biotrophic pathogens (Thomma et al., 1998; Glazebrook, 2005). Necrotrophic pathogens are typically characterised by having a broad host range, i.e. only few plant species display non-host resistance, and there exists no R-gene-mediated resistance (monogenic resistance, vertical resistance) against necrotrophic pathogens. In brief, defence reactions efficient in arresting

necrotrophs are scarce, which is especially the case for *B. cinerea* and *E. chrysanthemi*, the two pathogens used in this study. An obvious question arising is what makes *sitiens* display high levels of resistance to these pathogens. It was demonstrated earlier that basal tomato ABA levels have a suppressive effect on salicylic acid-responsive defence signalling (Audenaert et al., 2002a), which is striking because SA-responsive defence is generally considered to be effective against biotrophs and not against necrotrophs. Together, the successful resistance against a broad range of necrotrophic pathogens in *sitiens* is an uncommon phenomenon and the primary objective of this work was to identify and characterise the defence responses in *sitiens* that are responsible for the high level of resistance against *B. cinerea* and *E. chrysanthemi*.

In chapter 1 we present an overview of the present knowledge about the influence of ABA on plant pathogen-interactions. Although reports on ABA affecting pathogen defence are relatively uncommon, their number has increased significantly over the past few years, resulting in important progress that has been made in understanding the different functions of ABA in plant defence. We discuss the modes of ABA on different pathogen defence responses, such as the interaction with other plant hormones, phenylpropanoid biosynthesis, callose deposition, stomatal closure and reactive oxygen species (ROS) production, and we address the function of ABA as an important regulator in the integration of different plant stress responses.

In chapters 2 and 3 we examine the defence responses of *sitiens* to *B. cinerea*. By use of TOM1 microarrays we compared the expression of more than 8000 unigenes between *sitiens* and wild-type tomato before and after inoculation with *B. cinerea*. Furthermore, we performed a histochemical and microscopical comparison of the defence reactions in both tomato genotypes. The combined results demonstrate an important role for ROS-mediated cell wall fortification in *sitiens* defence against *B. cinerea*. In general, it is believed that ROS-formation is only effective in arresting biotrophic pathogens and positively influences necrotrophic tissue colonisation. The unexpected effectiveness of ROS-accumulation in arresting the necrotroph *B. cinerea* is elaborately discussed.

Callose deposition is an ABA-regulated plant defensive reaction considered to mediate resistance in different plant-pathosystems. In chapter 4 we assess the effect of ABA on callose formation in tomato and its importance in resistance to *B. cinerea*. We found that callose deposition is negatively affected by ABA deficiency. However, callose formation does not play a role in the high level of resistance present in *sitiens*, but seems to be involved in basal resistance of wild-type tomato to *B. cinerea*.

The role of ABA in the interaction of tomato with *E. chrysanthemi* is established in

chapter 5. Elevation and reduction of plant ABA content seem to have great effects in mediating disease susceptibility and resistance, respectively. We show that ROS-mediated cell wall fortification also has an important function in *sitiens* resistance to this pathogen. In addition, we expand our knowledge in this chapter on the elicitation of *sitiens* defence responses, as we demonstrate the importance of plant cell wall breakdown in the defence activation of *sitiens*.

In chapter 6 we explore the hypothesis that ABA-deficiency in *sitiens* results in differential recognition and/or early signalling events within the cell wall, leading to an efficient cell wall fortification response. By the use of immunological microscopy we found differences between *sitiens* and wild-type in content and composition of pectin and in distribution of arabinogalactan proteins. The possible implications of these structural differences on defence activation and early defence signalling are discussed.

In the final chapter 7 we provide general conclusions and discuss the applications of this work and suggest future research directions.

Chapter

1

Literature review: Global Switches and Fine-Tuning - ABA Affects Plant Pathogen Defence

Bob Asselbergh, David De Vleesschauwer and Monica Höfte

Plants are obliged to defend themselves to a wide range of biotic and abiotic stresses. Complex regulatory signalling networks mount an appropriate defence response depending on the type of stress that is perceived. In response to abiotic stresses such as drought, cold and salinity, the function of abscisic acid (ABA) is well documented: elevation of plant ABA levels and activation of ABA-responsive signalling result in regulation of stomatal aperture and expression of stress-responsive genes. In response to pathogens, the role of ABA is more obscure and is a research topic that has long been overlooked. This paper aims to evaluate and review the reported modes of ABA action on pathogen defence and highlight recent advances in deciphering the complex role of ABA in plant-pathogen interactions. In the majority of reports, elevated or basal ABA levels are associated with increases in disease susceptibility. As possible mechanisms of ABA-induced susceptibility, a suppressive effect of ABA on phytoalexin accumulation, salicylic acid-mediated defence responses, ROS-mediated cell wall fortification and jasmonate/ethylene-responsive defence responses have been proposed. The strong negative effects of ABA on defence responses and on disease phenotypes are indicative for an ABA-controlled global shift in priority towards biotic or abiotic stress response. This is also reflected in studies that implicate ABA as an essential virulence factor of bacterial and fungal plant pathogens: it was reported that elevation of *in planta* ABA levels and activation of ABA signalling are important to establish disease in some plant-pathosystems and to suppress plant defence responses in symbiotic interactions between plants and beneficial micro-organisms. In contrast, some studies present a positive function for ABA in defence to biotic stress: stimulation of callose deposition, jasmonate-mediated defence responses and stomatal closure in order to prevent pathogen entry were proposed to mediate ABA-dependent resistance. Furthermore, there are a fast-growing number of reports that characterise antagonistic and synergistic interactions between abiotic and biotic stress responses. A complex picture is emerging with ABA functioning as an essential component in integrating and fine-tuning abiotic and biotic stress response signalling networks.

1. INTRODUCTION

The capacity of plants to cope with the constant threat of a variety of plant pathogens demonstrates the efficiency of their defensive machinery. Plants possess pre-formed physical and biochemical barriers. When these constitutive defenses are overcome by a pathogen, recognition leads to a complex signaling cascade of inducible defense responses. The phytohormones, salicylic acid (SA), jasmonate (JA) and ethylene (ET) were shown to modulate these signaling pathways. Instead of forming isolated hormonally controlled signaling cascades, complex regulatory signaling networks with frequent cross-talk mount an appropriate defense response depending on the type of pathogenic stimuli that is present (Glazebrook, 2005; Thomma et al. 2001, Lorenzo and Solano, 2005, van Loon et al. 2006). Furthermore, there is frequent cross-talk between the signaling networks controlling the responses to abiotic stresses (Fujita et al. 2006; Mauch-Mani and Mauch, 2005). The use of shared components in biotic and abiotic stress responses is rationalized by economical use of biochemical resources, whereas antagonistic relationships between different stress responses result from the plants' need to activate an appropriate response to the type of stress that is encountered.

The phytohormone abscisic acid (ABA) not only regulates plant developmental processes such as seed maturation, dormancy, inhibition of germination, photoregulation, inhibition of lateral root formation, senescence and flowering inhibition, but also has a primary function in response to salt, drought, osmotic and cold stress (Finkelstein et al. 2002; Finkelstein and Rock, 2002). In addition to this well-studied function in the response to abiotic stress, a fast growing number of studies have demonstrated that ABA is also prominently involved in the response to pathogens and is implicated in the integration of different stress response signaling networks. However, to date our knowledge regarding the functions of ABA in response to pathogens is still very fragmentary. ABA was reported to play an ambivalent role in pathogen defense and several putative mechanisms were proposed (reviewed by Mauch-Mani and Mauch, 2005). This paper aims to evaluate and review the reported modes of ABA action on pathogen defense and highlight recent advances in deciphering the complex role of ABA in plant-pathogen interactions.

2. ABA AS A NEGATIVE REGULATOR OF PATHOGEN DEFENCE

Several lines of evidence point to ABA as a negative regulator of pathogen defence (Table I-I). Firstly, it was shown for different hosts that application of exogenous ABA increases disease susceptibility. ABA-pre-treatment increases susceptibility of barley, potato, rice, soybean, Arabidopsis, tomato and tobacco to various pathogens (Henfling et al., 1980; Salt et al., 1986; Ward et al., 1989; Edwards, 1983, Mohr and Cahill, 2003, Koga et al., 2004, Asselbergh et al., 2008). To our knowledge, no study has reported a direct effect of ABA on pathogen growth or performance *in vitro*, indicating that ABA interferes with plant defence. Secondly, mutations that disturb ABA biosynthesis were shown to increase resistance. ABA-deficient mutants of tomato are more resistant to *Botrytis cinerea* (Audenaert et al., 2002a), *Oidium neolycopersici* (Achuo et al., 2006), *Pseudomonas syringae* (Thaler and Bostock, 2004), *Erwinia chrysanthemi* (Asselbergh et al., 2008; results presented in chapter 5) and *Sclerotinia sclerotiorum* (Rotthier, 2004). In Arabidopsis, ABA-deficiency leads to reduced susceptibility to *Hyaloperonospora parasitica* (Mohr and Cahill, 2003), *Pseudomonas syringae* (de Torres-Zabala et al., 2007) and *Fusarium oxysporum* (Anderson et al., 2004). Thirdly, mutations that cause ABA-insensitivity by disturbing ABA signalling were shown to increase disease susceptibility. For example, Arabidopsis *abi1* and *abi2* mutants carrying non-functional ABI1 or ABA2 protein phosphatases 2C were shown to be more resistant to *Pseudomonas syringae* (de Torres-Zabala et al., 2007) and to *Plectosphaerella cucumerina* (Hernández-Blanco et al., 2007). Fourthly, application of ABA biosynthesis inhibitors can decrease susceptibility. Treatment of soybean with norflurazon decreased *Phytophthora sojae* lesion size (McDonald and Cahill, 1999) and treatment of rice with fluridon prevented cold-induced susceptibility (Koga et al., 2004). Fifthly, abiotic stress treatments, which are known to increase ABA content and to activate ABA signalling, increase susceptibility in some plant-pathosystems. For example, salt- or drought-stressed tomato displayed increased symptom severity caused by *Phytophthora parasitica* (Ristaino et al. 1988) and *Pseudomonas syringae* (Thaler and Bostock, 2004) and drought stress increased susceptibility of Arabidopsis to an avirulent isolate of *Pseudomonas syringae* (Mohr and Cahill, 2003). Finally, ABA measurements during the early stages of pathogen infection reveal an increase of ABA during compatible interactions, whereas ABA levels drop during incompatible interactions. A localised decrease in soybean ABA concentration occurred within 4 h after inoculation with an

incompatible isolate of *Phytophthora sojae*, but was absent during compatible interactions (Cahill and Ward, 1989). In wheat (*Triticum aestivum* L.) infected with powdery mildew (*Erysiphe graminis* f. sp. *tritici*), endogenous ABA levels increased in a susceptible cultivar and not in a resistant cultivar (Nikitina and Talieva, 2001).

2.1 Mechanisms of ABA-induced susceptibility

2.1.1. Suppression of PAL activity, secondary metabolites and SA accumulation

A possible mechanism that can explain the negative impact of ABA on pathogen defence is the suppression of phenylalanine ammonia lyase (PAL) activity by basal or elevated ABA levels (Ward et al., 1989; McDonald and Cahill, 1999; Audenaert et al., 2002a). PAL is a key enzyme in the early steps of the phenylpropanoid biosynthetic pathway, leading to the production of secondary antimicrobial metabolites, including phytoalexins and phytoanticipins. Incompatible interactions between soybean and *Phytophthora sojae* were marked by a sharp increase in PAL activity within 4 hpi, which was not present in compatible interactions and could be suppressed by exogenous ABA application (McDonald and Cahill, 1999). Reversely, artificial reduction of ABA levels with norflurazon during inoculation with compatible isolates elevated PAL activity and led to the formation of incompatible lesions (McDonald and Cahill, 1999). It was shown earlier in the same plant-pathosystem that ABA suppresses PAL activity at the transcriptional level (Ward et al., 1989). Furthermore, resistance in this interaction was correlated with the accumulation of the phenylpropanoid-derived compound glyceollin, which could be suppressed by ABA treatment, and was not correlated with lignin deposition nor with the presence of a hypersensitive response (HR) (Mohr and Cahill, 2001). These data show that plant ABA levels can determine the outcome of a plant-pathogen interaction by controlling the accumulation of phytoalexin production through regulation of phenylpropanoid biosynthesis. Besides leading to the formation of antimicrobial secondary metabolites, the phenylpropanoid pathway is involved in the synthesis of the plant defence hormone SA. In Arabidopsis, gene-for-gene resistance to *Pseudomonas syringae* is SA-dependent and application of exogenous ABA prevents the accumulation of SA and suppresses resistance (Mohr and Cahill, 2007). Transcriptome analysis confirmed the ABA-mediated suppression of genes in the early steps of the phenylpropanoid pathway including PAL and 4-coumarate/CoA ligase (Mohr and Cahill, 2007). In tomato, it was shown that the ABA-deficient *sitiens* mutant is hypersensitive to the SA analogue BTH and displays a hyperinduction of PAL activity after pathogen attack

(Audenaert et al., 2002a). Comparison of the transcriptome of *sitiens* and wild-type tomato confirmed transcriptional activation of the phenylpropanoid biosynthetic pathway in *sitiens*, and also showed an increased accumulation of SA-inducible defence-related transcripts such as PR1 both prior to and quickly after inoculation with *B. cinerea* (Asselbergh et al., 2007; results presented in chapter 2). A higher basal PR1 mRNA accumulation was also detected in other ABA-deficient tomato mutants (Thaler and Bostock, 2004). In addition, down-regulation of β-1,3-glucanase transcripts, another SA-inducible PR protein (PR2), was detected in tobacco cell cultures treated with ABA (Rezzonico et al., 1998). Together, these results indicate that exogenous ABA application can suppress SA accumulation and SA-inducible defence transcript accumulation, whereas a decrease in endogenous ABA results in constitutive activation and hyperinduction of SA-dependent defences.

Besides the repression on phenylpropanoid biosynthesis, other mechanisms were suggested for the suppressive effect of ABA on SA-inducible gene expression. Adie et al. (2007) proposed that ABA-SA antagonism could also be explained by an indirect effect based on the ABA-mediated induction of JA biosynthesis (See also paragraph 3.1.3). Another conceivable justification for ABA-SA antagonism lies in the positive effect of ABA on callose formation (see paragraph 3.1.2). As callose was shown to block SA-inducible defence responses (Nishimura et al., 2003), the action of ABA on SA-dependent responses could be partly due to enhancement of callose deposition.

Table I-I: Plant-pathogen interactions with negative effects of ABA on defence responses.

Host plant	Pathogen	Proposed mode of ABA action on defence responses[a]	ABA decrease		inactivation of ABA signaling		ABA increase		reference
			method - effect on resistance[a,b,c]		method - effect on resistance[a,b,d]		method - effect on susceptibility[a,b,e]		
Tomato	*Botrytis cinerea*	suppression of SA responses, PAL activity, hydrogen peroxide accumulation and cell wall fortification	bm: *sitiens, notabilis, flacca;* bi: fluridone	++	ND		ex, ss, ds	+	Audenaert et al., 2002; Asselbergh et al., 2007; Achuo et al., 2006
Tomato	*Erwinia chrysanthemi*	suppression of ROS accumulation and cell wall fortification	bm: *sitiens*	++	ND		ex	++	Asselbergh et al., 2008
Tomato	*Oidium neolycopersici*	ND	bm: *sitiens*	+	ND		ex	=	Achuo et al., 2006
Tomato	*Pseudomonas syringae*	suppression of SA responses	bm: *sitiens, flacca*	+	ND		ss	+	Thaler & Bostock, 2004
Tomato	*Sclerotinia sclerotiorum*	ND	bm: *sitiens*	++	ND		ND		Asselbergh and Höfte, unpublished results
Arabidopsis	*Fusarium oxysporum*	suppression of JA/ET responses	bm: *aba2-1*	+	ND		ND		Anderson et al., 2004
Arabidopsis	*Botrytis cinerea*	ND	bm: *aao3-2* , *aba2-12*	++	im: *abi4-1*	+	ND		Adie et al., 2007
Arabidopsis	*Pseudomonas syringae*	suppression of basal defence responses	bm: *aao3*	++	im: *abi1-1* , *abi2-1* , *35S::HAB1*	++	ex	+	de Torres-Zabala et al., 2007
Arabidopsis	*Pseudomonas syringae*	suppression of SA responses and lignin accumulation	bm: *aba1-1*	=	im: *abi1-1*	=	ex, ds	++	Mohr & Cahill, 2003, Mohr & Cahill, 2007
Arabidopsis	*Hyaloperonospora parasitica*	ND	bm: *aba1-1*	++	im: *abi1-1*	=	ex, ds	=	Mohr & Cahill, 2003
Arabidopsis	*Plectosphaerella cucumerina*	ND	bm: *aba1-6*	++	im: *abi1-1* , *abi2-1*	++	ND		Hernández-Blanco et al., 2007

(Table continues on next page.)

Table I-I (continued)

Host plant	Pathogen	Proposed mode of ABA action on defence responses[a]	ABA decrease		inactivation of ABA signaling	ABA increase		reference
			method - effect on resistance[a,b,c]		method - effect on resistance[a,b,d]	method - effect on susceptibility[a,b,e]		
Barley	*Erysiphe graminis* f. sp. *hordei*	ND	ND		ND	ex	++	Edwards, 1983
Potato	*Phytophthora infestans*	unknown	ND		ND	ex	++	Henfling et al., 1980
Potato	*Cladosporium cucumerinum*	ND	ND		ND	ex	++	Henfling et al., 1980
Rice	*Magnaporthe grisea*	suppression of 'whole plant-specific resistance'	bi: fluridone	+	ND	ex, cs	+	Koga et al., 2004
Tobacco	*Hyaloperonospora tabacina*	ND	ND		ND	ex	++	Salt et al., 1986
Tobacco	*Ralstonia solanacearum*	ND	ND		ND	ex	+	Steadman & Sequira, 1970
Wheat	*Erysiphe graminis* f. sp. *tritici*	ND	ND		ND	ND		Nikitina & Talieva, 2001
Soybean	*Phytophthora sojae*	suppression of PAL activity, glyceollin accumulation	bi: norflurazon	++	ND	ex	++	Ward et al., 1989; McDonald & Cahill, 1999; Mohr & cahill, 2001

[a]: ND: not determined; [b]: =: no significant effect, +: moderate effects, ++: relatively strong effects, based on the authors' evaluation of disease indexes in each report;
[c]: bm: biosynthesis mutants; bi: biosynthesis inhibition; [d]: im: insensitive mutants; [e]: ex: exogenous application; ds: drought stress; cs: cold stress; ss: salt stress.

2.1.2. Suppression of ROS accumulation

The importance of reactive oxygen species (ROS) accumulation during pathogen defence is well documented (Lamb and Dixon, 1997; Wojtaszek, 1997; Apel and Hirt, 2004; Torres and Dangl, 2005). Analysis of the resistance mechanisms of the ABA-deficient *sitiens* tomato mutant revealed the importance of rapid and extensive hydrogen peroxide accumulation in arresting the necrotrophic pathogens *B. cinerea* and *E. chrysanthemi* (Asselbergh et al., 2007; Asselbergh et al., 2008; results presented in chapters 2 and 5). Extracellular hydrogen peroxide accumulation and activation of peroxidases in *sitiens* caused rapid cell wall modification upon pathogen inoculation by protein cross-linking and incorporation of phenolic compound, causing the arrest of pathogen progression. Pathogen susceptibility could be restored by application of exogenous ABA or by pharmacologic disruption or removal of hydrogen peroxide accumulation (Asselbergh et al., 2007). Extensive ROS accumulation and increases in peroxidase activity as a result of ABA-deficiency is consistent with the hyperinduction of SA-inducible defences in ABA mutants (Asselbergh et al., 2007, Audenaert et al., 2002a). Many studies report on the relationship between ROS and SA in biotic stress responses. It is believed that ROS and SA work together in a self-amplifying system in establishing systemic acquired resistance (Alvarez et al., 1998; Van Camp et al., 1998; Van Breusegem et al., 2001; Durrant and Dong, 2004). This model is corroborated by ABA deficiency-triggered potentiation of both ROS- and SA-mediated defence. On the other hand, ROS are important messengers in ABA-mediated stress responses. ROS are key signals in regulating stress-adaptive ABA responses (Pastori and Foyer, 2002) and ABA-signalling in guard cells requires ROS formation to interact with Ca-channels to induce stomatal closure (Kwak et al., 2006; Li et al., 2006). Interestingly, guard cell ABA activates ROS-generating NADPH oxidases (Kwak et al., 2006), which are also necessary for ROS-production during pathogen defence (Torres and Dangl, 2005). Furthermore, ROS-production resulting in stomatal closure could be induced by application of plant cell wall degradation products (oligogalacturonides) (Lee et al., 1999) and it was shown that hydrogen peroxide-dependent defence responses in *sitiens* are most likely also elicited by oligogalacturonides (Asselbergh et al., 2008; see chapter 5). Together, it seems that at least some components for ROS-generation and ROS-signalling activation are common for ABA-responsive abiotic stress signalling in guard cells and for hyperactivation of pathogen defence in ABA-deficient plants. The mechanism by which ABA-deficiency in *sitiens* leads to rapid extensive ROS formation upon pathogen attack is at present conjectural.

2.1.3. Suppression of JA/ET responsive defences

The antagonistic effect of ABA on JA/ET pathogen defence signalling was proposed as an alternative mechanism for ABA to negatively influence pathogen defence (Andersen et al., 2004; Mauch-Mani and Mauch, 2005). By using *PDF1.2*, *b-CHI* and *HEL* transcript accumulation as markers for JA/ET responsive gene expression in Arabidopsis, it was shown that both basal and JA/ET-induced defence gene expression was suppressed by exogenous ABA and was upregulated in ABA-deficient *aba1* and *aba2* mutants, the latter resulting in increased resistance to *Fusarium oxysporum* (Andersen et al., 2004).

During wound and pathogen stress, positive and negative interactions between JA and ET signalling pathways are essential for the establishment of suitable plant defense responses. During pathogen attack, ET and JA cooperate through transcriptional induction of ethylene response factor 1 (ERF1), which results in activation of pathogen response genes such as *PDF1.2*, *b-CHI* and *HEL*. In response to wounding, JA activates the transcription factor AtMYC2, leading to wound stress-specific gene activation (such as *VSP* and *lox*). Repression of pathogen response genes by AtMYC2 and repression of wound response genes by ERF1 constitute important points of cross-talk between the two signalling pathways (Lorenzo and Solano, 2005). AtMYC2 expression was shown to be activated by ABA (Lorenzo et al., 2004) and it was suggested that ABA precedes JA in the activation of AtMYC2-mediated wound responses (Lorenzo and Solano, 2005). Therefore, AtMYC2 functions as a mediator of ABA to repress JA/ET-induced pathogen response (Andersen et al., 2004). However, the suppression of JA/ET-induced pathogen response genes by ABA can not be solely attributed to AtMYC2, as a suppressive effect remained in an *Atmyc2* mutant background (Andersen et al., 2004). Antagonistic interactions between ET and ABA signaling form an alternative mechanism of ABA to repress the JA/ET-induced pathogen response. For example, it was shown that ET treatment quickly induces activation of ABI1 and ABI2, two negative regulators of ABA signaling (De Paepe et al., 2004). Furthermore, by using ABA and ET signalling mutants, it was shown that the relationship between the two phytohormones is mutually antagonistic in vegetative tissues (Anderson et al., 2004).

Recent evidence suggests that the antagonistic ABA-ET crosstalk might be modulated by the ethylene-responsive element binding factor AtERF4 (Yang et al., 2005). AtERF4 is a transcriptional repressor whose expression is induced by ABA, ET, or JA exogenous treatment, while its overexpression leads to the inhibition of GCC box-containing defense genes, ethylene insensitivity, and decreased ABA sensitivity. In rice, exogenous ABA treatment has been

shown to decrease endogenous ET levels and consequently increase host susceptibility to *Magnaporthe oryzae* (Yang, 2007). Elegant research by Yinong Yang's group revealed that the suppressive effect of ABA on the ET signaling pathway is mediated by the *OsMPK5* gene. *OsMPK5* encodes an ABA-inducible mitogen-activated protein kinase that positively regulates endogenous ABA level and abiotic stress tolerance but negatively modulates endogenous ET level, pathogenesis-related gene expression and resistance to *Magnaporthe oryzae* (Xiong and Yang, 2003; Yang, 2007).

Collectively, it appears that the cross-talk of ABA with JA and ET signaling pathways occurs at multiple convergence points and that ABA acts negatively on the specific set of pathogen response genes that are controlled synergistically by ET and JA.

2.2 ABA regulates disease phenotypes in a forceful manner

One common trend that is observed among the different interactions in which ABA negatively influences disease resistance, are the relatively strong effects of ABA on disease phenotypes. For example, ABA-pre-treatment of potato slices altered the interaction with an incompatible isolate of *Phytophthora infestans* to obtain disease symptoms indistinguishable from a compatible interaction. Furthermore, the same ABA treatment allowed development of *Cladosporium cucumerinum*, normally not a pathogen of potato (Henfling et al., 1980). In tomato, occurrence of maceration caused by *Erwinia chrysanthemi* was strongly reduced in the ABA-deficient *sitiens* mutant and spreading maceration symptoms were completely absent. This drastic reduction of disease symptoms is remarkable, considering that sources of resistance to this broad-spectrum pathogen are rare (Asselbergh et al., 2008). Fast and extensive extracellular hydrogen peroxide accumulation in *sitiens* was shown to be essential in establishing resistance to both *E. chrysanthemi* and *B. cinerea* (Asselbergh et al., 2007; Asselbergh et al., 2008; see paragraph 2.1.2). Interestingly, production of ROS is normally not effective against necrotrophic pathogens such as *B. cinerea*, or can even facilitate necrotrophic tissue colonisation (Govrin and Levine, 2000). The effective arrest of *B. cinerea* by a timely hyperinduction of hydrogen peroxide-dependent defences in *sitiens* illustrates the strong effect of ABA-deficiency on the defensive capacity towards pathogens (Asselbergh et al., 2007). Analysis of *sitiens* defence activation after inoculation with *E. chrysanthemi* pathogenicity mutant strains and with *E. chrysanthemi* culture filtrate, demonstrated that defences are activated by *E. chrysanthemi* type II secreted proteins, which mainly consist of pectinases

(Asselbergh et al., 2008). *E. chrysanthemi* pectinolytic cell wall degradation causes the release of plant cell wall oligogalacturonides, which are known and potent endogenous elicitors of plant pathogen defences (Ridley et al., 2001). Defence activation by endogenous elicitors that are pathogen non-specific is consistent with the broad spectrum of pathogens that is unsuccessful in efficiently infecting *sitiens* plants (Asselbergh et al., 2008). These observations indicate that ABA has the capacity to negatively affect a broad range of plant pathogen interactions in an extreme and forceful manner.

2.3 ABA acts as a virulence factor of plant pathogens

The potency of ABA to suppress pathogen defence responses is exemplified by the exploitation of ABA as a virulence factor by plant pathogens. A recent study elegantly demonstrated that *Pseudomonas syringae* type III-secreted effectors (T3SE) target the Arabidopsis ABA signalling pathway to cause disease (de Torres-Zabala et al., 2007). Exogenous ABA decreases resistance in this interaction, and in addition, ABA insensitivity (in *abi1.1* and *abi2.1* mutants) or hypersensitivity (in *abi1.sup7* and *abi2.sup5* mutants) led to restriction or enhanced bacterial multiplication, respectively. Comparison of the Arabidopsis transcriptome during infection with wild-type and T3SE-negative *P. syringae* mutants revealed the overrepresentation of ABA-dependent gene expression in response to T3SE. The induction of ABA signalling by T3SE was represented by the upregulation of known ABA responsive genes, by the presence of ABA-responsive elements (ABRE) in the promoter regions of T3SE-induced genes and by the similarity between the transcriptomic profiles after T3SE-induction and ABA treatment. The upregulation of the ABA biosynthetic gene *NCED3* revealed the stimulation of ABA biosynthesis by T3SE, which was confirmed by ABA measurements. Finally, transgenic expression of the conserved *P. syringae* effector AvrPtoB induced *NCED3* expression and elevated ABA levels *in planta* (de Torres-Zabala et al., 2007). These findings demonstrate that bacterial effector-mediated elevation of plant ABA biosynthesis and signalling is a major virulence strategy, which leads to the suppression of defence responses.

In addition to regulating plant ABA biosynthesis, micro-organisms are known to synthesise ABA themselves. ABA is produced by different types of fungi, including ascomycetes, basidiomycetes and zygomycetes (Crocoll et al., 1991, Dörffling et al., 1984). To our knowledge, all fungal ABA-producing strains isolated so far are associated with plants. ABA biosynthesis in fungi differs from plant biosynthesis and is mediated through a direct

pathway via farnesyl diphosphate (Hirai et al., 2000). The fungal ABA biosynthetic pathway is biochemically best characterised in *Cercospora* species (Oritani and Kiyota, 2003) and in *B. cinerea* (Siewers et al., 2006). Recently, it was found that ABA biosynthetic genes in *B. cinerea* are organised in a gene cluster consisting of at least four co-regulated genes (Siewers et al., 2006). In the tomato - *B. cinerea* interaction, ABA-deficiency results in a decrease of the number of spreading *B. cinerea* lesions (Audenaert et al., 2002a; Asselbergh et al., 2007), whereas application of exogenous ABA increases the rate of symptom spreading (Kettner and Dörffling, 1995). By measuring the ABA levels and fungal ABA precursors in wild-type and ABA-deficient tomato infected with ABA-producing and ABA non-producing strains of *B. cinerea*, Kettner and Dörffling (1995) found that at least four processes elevate the level of ABA in tomato infected with *B. cinerea*: host ABA biosynthesis is stimulated by the fungus; ABA and its precursor are released by the fungus; fungal ABA biosynthesis is stimulated by the presence of the host; host ABA catabolism is inhibited by the fungus (Kettner and Dörffling, 1995). These findings, together with the capacity of phytopathogenic fungi from taxonomically unrelated groups to produce ABA, indicate that elevation of host ABA levels can function as a general pathogenic strategy to suppress host defences. Assessment of the pathogenicity of ABA- and ABA precursor-negative fungal mutants, such as those described by Siewers et al. (2006), could further elucidate the function of ABA in pathogen virulence.

Interesting information regarding a suppressive role of ABA on plant defence can also be derived from the symbiotic interactions of plant roots with arbuscular mycorrhizal (AM) fungi and nitrogen-fixating bacteria. It can be contemplated that suppression of plant defences by ABA functions as an important strategy to establish these symbiotic interactions. First, the plant defence response during symbiotic root colonisation is characterised by only a localised, weak and transient activation, which implicates the suppression of pathogen defence responses (García-Garrido and Ocampo, 2002). General or conserved mechanisms of disease suppression seem to be involved, given the similarities between AM infection and rhizobial colonization (Albrecht et al., 1999) and the fact that in general AM fungi show little or no specificity, with the factors that determine whether or not colonization occurs appearing to depend solely on the genotype of the host plant (Koide and Schreiner, 1992). Second, the defence responses that were most clearly shown to be transiently expressed and subsequently suppressed to allow AM colonisation were the accumulation of SA and the increase in peroxidase activity (Blilou et al. 1999; Blilou et al., 2000; García-Garrido and Ocampo, 2002), defence responses known to be suppressed by ABA. Third, ABA-producing strains were detected for *Glomus* species of AM fungi (Esch et al., 1994; Bothe et al., 1994), *Anabaena* filamentous cyanobacteria (Esch et al.,

1994) and root nodule *Rhizobium* species (Dangar and Basu, 1991). Fifth, AM colonisation is known to change plant hormone homeostasis (Hause et al., 2007), including increases in ABA content (Esch et al., 1994; Meixner et al., 2005). Finally, abiotic stress tolerance is strongly increased after AM colonisation (Smith and Read, 1997; Ruiz-Lozano, 2003), which presumably implies the involvement of enhanced ABA-mediated responses during AM colonisation.

A direct link between ABA and successful AM colonisation was recently provided in the tomato – *Glomus intraradices* interaction (Herrera-Medina et al., 2007). Colonisation of the ABA-deficient *sitiens* mutant was less frequent and arbuscule development was incomplete. Reversely, application of exogenous ABA increased AM colonisation in wild-type and mutant plants. The authors suggested that impairment of AM development in ABA-deficient mutants was at least partly attributable to the antagonistic interaction of ABA with ET (Herrera-Medina et al., 2007).

3. ABA AS A POSITIVE REGULATOR OF PATHOGEN DEFENCE

Several observations support the fact that ABA signalling can have a positive effect on pathogen defence (Table I-II). First, exogenous ABA can enhance resistance in some plant-pathosystems. ABA treatment was shown to increase the level of resistance in barley (*Hordeum vulgarum*), Arabidopsis and common bean (*Phaseolus vulgaris*) (Wiese et al., 2004; Dunn et al., 1990; Kaliff et al., 2007). Likewise, ABA-pretreated rice plants, yet displaying increased susceptibility to the leaf blast pathogen *Magnaporthe oryzae*, were recently shown to exhibit significantly enhanced levels of resistance to *Bipolaris oryzae* (De Vleesschauwer and Höfte, unpublished results). Second, ABA-deficient and ABA-insensitive mutants are more susceptible to some pathogens. This was shown for Arabidopsis interacting with *Alternaria brassicicola*, *Pythium irregulare* (Adie et al., 2007), *Leptosphaeria maculans* (Kaliff et al., 2007) and *Ralstonia solanacearum* (Hernández-Blanco et al., 2007). Inhibiting ABA biosynthesis with norflurazon also reduced resistance to *Colletotrichum lindemuthianum* in bean (Dunn et al., 1990). Finally, gene expression during plant defence responses was found to depend largely on ABA-inducible genes in a number of resistant interactions. For example, of the genes upregulated in Arabidopsis cellulose synthase (cesa) mutants, which are more resistant to several pathogens of different lifestyles, over 50% is induced by ABA (Hernández-Blanco et al., 2007). Similarly, meta-analysis of Arabidopsis transcriptome profiles clustered the defence response to *Pythium irregulare* together with the responses to ABA-treatment and several forms of abiotic stress, demonstrating a role for ABA in defence against this pathogen (Adie et al., 2007).

When positive effects of ABA on defence responses are evaluated, special care should be taken to discriminate between direct and indirect effects on pathogen defence, especially during interactions with root rot and/or wilting pathogens. Since these types of pathogens impinge plant water balances or fluxes and cause severe dehydration stress, ABA-induced abiotic stress responses to enhance dehydration stress tolerance will be activated and can thereby reduce disease symptoms. ABA-induced stomatal closure to limit evaporation water loss and to counteract wilting symptoms is a nice example of an indirect positive effect of ABA on pathogen defences, because it is principally an abiotic stress response and not a response to biotic stress.

Table I-II: Plant-pathogen interactions with positive effects of ABA on defence responses.

Host plant	Pathogen	Proposed mode of ABA action on defence responses[a]	ABA decrease		inactivation of ABA signaling		ABA increase		reference
			method - effect on susceptibility[a,b,c]		method - effect on susceptibility[a,b,d]		method - effect on resistance[a,b,e]		
Arabidopsis	*Pythium irregulare*	stimulation of JA biosynthesis	bm: *aao3-2* , *aba2-12*	++	im: *abi4-1*	++	ND		Adie et al., 2007
Arabidopsis	*Alternaria brassicicola*	stimulation of JA biosynthesis	bm: *aao3-2* , *aba2-12*	++	im: *abi4-1*	++	ND		Adie et al., 2007
Arabidopsis	*Alternaria brassicicola*	priming for callose deposition	bm: *aba1-5*	+	im: *abi4-1*	+	ex	+	Ton & Mauch-Mani, 2004
Arabidopsis	*Plectosphaerella cucumerina*	priming for callose deposition	bm: *aba1-5*	=	im: *abi4-1*	=	ex	+	Ton & Mauch-Mani, 2004
Arabidopsis	*Sclerotinia sclerotiorum*	stomatal closure	bm: *aba2-1*	++	im: *abi1-1* (+), *abi2-1* (=), *abi3-1* (+)	+/=	ND		Guimarães & Stotz, 2004
Arabidopsis	*Pseudomonas syringae*	stomatal closure in innate immunity pathway	bm: *aba3-1*		gcsm: *coi1-20* , *ost1-2*		ex		Melotto et al., 2006
Arabidopsis	*Ralstonia solanacearum*	signaling in irx-mediated resistance (leading to antimicrobial compounds)	bm: *aba1-6*	++	im: *abi1-1* , *abi2-1*	++	ND		Hernández-Blanco et al., 2007
Arabidopsis	*Leptosphaeria maculans*	signaling in RLM pathway leading to callose dependent and -independent resistance	bm: *aba1-3, aba2-1* , *aba3-1*	+	im: *abi1-1* (+), *abi4-1* (++), *abi2-1* (=), *abi3-1* (+), *abi5-1* (=)	+/++/=	ex	=	Kaliff et al., 2007
Rice	*Bipolaris oryzae*	priming for MPK5-mediated repression of ethylene signaling	bi: fluridone	=	ND		ex	+	De Vleesschauwer and Höfte, unpublished results
Bean	*Colletotrichum lindemuthianum*	ND	bi: fluridone	+	ND		ex	+	Dunn et al., 1990
Lily	*Botrytis elliptica*	stomatal closure in probenazol-induced resistance	ND		ND		ex	+	Lu et al., 2007
Barley	*Blumeria graminis f. sp. hordei*	ND	ND		ND		ex, os, ps	+	Wiese et al., 2004
tobacco	Tobacco Mosaic Virus	Stimulation of callose deposition	ND		ND		ex	+	Whenham et al., 1986; Fraser & Whenham, 1989; Balazs et al., 1973; Rezzonico et al., 1998

[a]: ND: not determined; [b]: =: no significant effect; +: moderate effects; ++: relatively strong effects, based on the authors' evaluation of disease indexes in each report; [c]: bm: biosynthesis mutants; bi: biosynthesis inhibition; [d]: im: insensitive mutants; gcsm: guard cell signaling mutants; [e]: ex: exogenous application; os: osmotic stress; ps: proton stress.

3.1. Mechanisms of ABA-induced resistance

3.1.1. Stomatal closure

In addition to the indirect effect of ABA signalling by inducing stomatal closure to limit water loss and thereby counteracting the development of disease symptoms, a biologically very relevant direct positive effect of ABA signalling on pathogen defence is by closing stomata to prevent pathogen invasion. It was recently shown that stomatal closure is is integral to pre-invasion pathogen-associated molecular pattern (PAMP)-induced innate immunity to bacteria (Melotto et al., 2006; Underwood et al., 2007). Stomata close upon recognition of plant pathogens, human pathogens (plant non-pathogens) and isolated PAMP molecules, a process that requires ABA signalling in guard cells and ABA biosynthesis (Melotto et al., 2006). Moreover, it was elegantly demonstrated that *Pseudomonas syringae* pv. *tomato* needed the virulence factor coronatine in order to enter internal leaf tissue by inhibiting ABA-induced stomatal closure. Coronatine, a JA-mimic, counteracts PAMP-induced stomatal closure downstream of ABA, but requires functional COI1 signalling. Interestingly, PAMP-induced stomatal closure was compromised in SA-deficient transgenic *nahG* plants and SA-biosynthetic mutant *eds16-2* plants, indicating that defence through stomatal closure is an integral part of the SA-regulated innate immune system (Melotto et al., 2006). These results show that counteracting ABA-dependent signalling in guard cells is a pathogenic strategy to overcome pre-invasion SA-regulated innate immunity. This is in sharp contrast to the up-regulation of ABA signalling and ABA biosynthesis needed for post-penetration virulence (de Torres-Zabala, 2007) and the repression of SA accumulation and SA-dependent defence gene expression by ABA during infection (Mohr and Cahill, 2007), both in the same plant-pathosystem. Interestingly, stomatal defence and bacterial suppression of stomatal defence seem common phenomena in plant-bacterium interactions, as PAMPs also induce stomatal closure in tomato, which could also be modulated by *Pseudomonas syringae* pv. *tomato* (Melotto et al., 2006). Considering that in natural environments, bacterial and many fungal pathogens rely entirely on accidental wounds or natural plant openings such as stomata to enter internal plant tissues, the impact of stomatal defence on plant-pathogen interactions in nature can hardly be overestimated. It remains to be elucidated whether PAMP-induced ABA-signalling is limited to guard cells, or if ABA-induced signalling early upon pathogen recognition also occurs in other plant cell types.

In addition, it was shown earlier that the fungal toxin fusicoccin promotes stomatal

opening and antagonises ABA-induced stomatal closure (Marré, 1979). Also the fungal necrotrophic pathogen *Sclerotinia sclerotiorum* uses the virulence factor oxalate to prevent ABA-induced stomatal closure during infection (Guimarães and Stotz, 2004). Increased wilting and facilitation of hyphal emergence and secondary colonisation were proposed to result from the prevention of stomatal closure. The mechanism by which oxalate suppresses ABA-induced stomatal closure remains unknown (Guimarães and Stotz, 2004), and the elucidation of this mechanism will be further complicated by the multiple functions of oxalate in necrotrophic virulence (Van Kan, 2006). Nevertheless, ABA-induced stomatal closure is undoubtedly an important plant defence strategy towards pathogens.

3.1.2. Stimulation of callose deposition

Another positive effect of ABA on pathogen defence is by its ability to stimulate callose deposition. Callose is a β-1,3-glucan that is deposited in cell wall appositions (papillae) that can block pathogen entry (Aist, 1976). It was reported that both ABA signalling and callose formation are prerequisites for β-amino butyric acid (BABA)-triggered induced resistance to *Plectosphaerella cucumerina* and *Alternaria brassicicola* in Arabidopsis (Ton and Mauch-Mani, 2004). Treatment with exogenous ABA could mimic the effect of BABA and resulted in priming for callose and resistance to *P. cucumerina*. In addition, Arabidopsis resistance to *Leptosphaeria maculans* through the $RLM1_{col}$ pathway and to *Pythium irregulare* was shown to be partly mediated by ABA-dependent callose formation, next to callose-independent ABA-dependent resistance mechanisms (Kaliff et al., 2007; Adie et al., 2007). ABA treatment in barley also caused papillae-mediated resistance against *Blumeria graminis* f. sp. *hordei* (Wiese et al., 2004). In the interaction of tomato with *B. cinerea*, callose deposition was low in the ABA-deficient *sitiens* mutant and was not important for its resistant response. However, ABA-dependent callose formation was involved in basal defence of wild-type tomato (Asselbergh and Höfte, 2007; results presented in chapter 4). In contrast to these reports, a negative effect of ABA on callose deposition was shown in Arabidopsis challenged with *Pseudomonas syringae* pv. *tomato* (de Torres-Zabala et al., 2007). ABA-hypersensitivity (in *abi1-sup7* and *abi1-sup5* mutants) and exogenous ABA treatment strongly reduced callose deposition, while ABA-insensitive mutants (*abi1-1* and *abi2-1*) show augmented callose deposition (de Torres-Zabala et al., 2007).

It is noteworthy that in some cases ABA does not directly regulate callose deposition upon pathogen attack, but rather modulates the priming of its deposition (after BABA

treatment) (Flors et al., 2005). Also, the fact that BABA treatment enhances the capacity to resist abiotic stress (Ton et al., 2005), indicates that priming for callose deposition is mediated by ABA-responsive signalling components that are common for biotic and abiotic stress responses. This view is supported by a recent study, which shows that salt stress and BABA act synergistically in tomato to induce resistance to *Pseudomonas syringae* pv. *tomato* (Baysal et al., 2007).

The molecular mechanisms behind the modulation of callose by ABA remain to be elucidated. It was suggested that ABA could control callose deposition by regulating vesicle-mediated transport of callose synthase proteins. Transcriptional activation by ABA of specific N-ethyl-malmeimide-sensitive fusion protein attachment protein receptors (SNAREs) was speculated to direct callose synthase proteins to the site of pathogen attack (Flors et al., 2005). Alternatively, it was proposed earlier that ABA down-regulates β-1,3-glucanases, which use callose as a substrate (Rezzonico et al., 1998).

3.1.3. Stimulation of JA biosynthesis/signalling

Another feature that allows ABA to positively interact with pathogen resistance is by its positive interaction with JA-mediated defence responses. Although antagonistic interactions between JA and ABA have been reported (Moons et al., 1997; Lorenzo and Solano, 2005), these two hormones often act as positive regulators in the same signaling pathway. In Arabidopsis guard cells both JA and ABA induce stomatal closure by activation of identical secondary messengers, such as ROS, NO, Ca^{2+} permeable cation channels and S-type anion channels (Munemasa et al., 2007). During the wound response, high levels of ABA and JA accumulate locally and systemically to mediate wound-activated gene expression and it was suggested that ABA action precedes JA biosynthesis (León et al., 2001). Recently, Adie et al. (2007) demonstrated that ABA is an essential signal leading to JA biosynthesis with resultant activation of defence responses against the damping-off oomycete *Pythium irregulare*. Defence signalling against this pathogen relies partly on ET and SA, but is predominantly mediated by JA, with JA-insensitive *coi1* mutants showing extreme susceptibility. Transcriptome analysis of wild type, ET-, SA- and JA-related mutants (Col-0, *ein2-5*, *sid2-1* and *coi1-1* respectively) after infection with *P. irregulare* allowed the division in JA/ET/SA-dependent and JA/ET/SA-independent genes induced by *P. irregulare*. *P. irregulare*-induced JA/ET/SA-dependent genes were dominated by JA-responsive genes. Meta-analysis confirmed the dependence on JA-responsive genes as well as revealed high similarity of the *P. irregulare*-induced transcriptome

with the response to ABA. Promoter analysis of the JA/ET/SA-dependent *P. irregulare*-induced genes also revealed an overrepresentation of ABA-response elements. Furthermore, the *P. irregulare*-induced transcriptome independent of JA/ET/SA clustered together with the profiles of responses to ABA and abiotic stresses. ABA-deficient (*aba2-12*) and ABA-insensitive (*abi4-1*) mutants showed impaired JA biosynthesis and increased susceptibility upon *P. irregulare* infection (Adie et al., 2007). Together, these results elegantly show the requirement for ABA signalling to activate JA-dependent resistance to *P. irregulare*. Interestingly, this study also confirmed the down-regulation by ABA of a group of JA/ET-responsive genes, such as *PDF1-2*, *HEL* and *b-CHI*, which confirmed earlier findings (Anderson et al., 2004). However, the transcriptomic view showed that the major effect of ABA is the opposite, activating many ABA-specific and ABA/JA-related defence genes (Adie et al., 2007).

4. COMPLEXITY OF ABA-MEDIATED RESPONSES

The process of establishing and interpreting possible functions of ABA in plants is hindered by the complexity of ABA-mediated responses. ABA-dependent responses are regulated by controlling *de novo* ABA synthesis. The first steps of ABA biosynthesis occur in chloroplasts where cleavage of carotenoids by nine-cis epoxycarotenoid dioxygenases (NCEDs) is the rate-limiting step and transcriptional regulation of the NCEDs is the major control point of ABA biosynthesis. In addition, the level of ABA in plants is not only controlled by its synthesis, but also through its catabolism (Schwartz et al., 2003). However, ABA-regulated processes constitute more than a simple response to *in planta* bulk ABA concentrations (Wilkinson and Davies, 2002). During drought stress, instant stomatal closure is essential for the plants survival and is mediated by ABA-perception at the guard cells. Whereas severe drought stress that causes water deficit in the shoot is followed by a drastic increase in levels of intracellular leaf ABA, drought stress that is only perceived at the roots specifically increases apoplastic ABA in guard cells without influencing symplastic leaf ABA contents or leaf bulk apoplastic ABA (Wilkinson and Davies, 2002). This implicates not only the existence of ABA perception sites at different locations and the co-regulation of chemical and hydraulic signals, but also implies a whole-plant modulation of the ABA-signal, including differential xylem loading in the roots, ABA sequestration into a symplastic leaf reservoir and alteration of guard cell sensitivity to ABA (Wilkinson and Davies, 2002). In addition, drought stress-induced ABA

can stimulate primary root elongation while shoot growth decreases, leading to increased water absorption and reduced water loss, respectively (Sharp, 2002). These and other findings indicate that, even during a single stress response, different signalling mechanisms are required that can be stage, organ or cell specific.

One mechanism that can help to explain the diversity in ABA responses is the existence of different ABA receptors. Indeed, over the last few years, different research groups have characterised three proteins that each fulfil the biochemical requirements of an ABA receptor (stereospecific and saturable high affinity binding to one binding site). The Arabidopsis nuclear protein FCA (for flowering control protein A) is an RNA-binding protein and is required for ABA-signalling in controlling flowering and lateral root formation, but not in seed germination or the stomatal response (Razem et al., 2006). The Arabidopsis protein ABAR/CHLH (putative ABA receptor/Mg-chelatase H subunit) specifically binds ABA in chloroplasts, functions at the whole plant level and controls seed germination and stomatal movement (Shen et al., 2006). Finally, a G-protein coupled receptor was shown to perceive ABA at the cell surface and to mediate all known/tested ABA responses in Arabidopsis (Liu et al., 2007).

Downstream of ABA perception, the components of the complex signalling network include RNA-binding proteins (Hugouvieux et al., 2001), protein kinases (Osakabe et al., 2005), protein phosphatases (Leung et al., 1997) and multiple-type transcription factors (Finkelstein and Rock, 2002). In addition, a recent transcriptomic analysis that used ABA structural analogues to detect genes that are weakly induced by ABA estimated that about 14% of Arabidopsis genes are ABA-regulated (Huang et al., 2007). Furthermore, comparison of the ABA-regulated genes that were identified in other studies (Hoth et al., 2002; Seki et al., 2002; Leonardt et al., 2004) suggests that the full potential of ABA-responsive gene regulation has not yet been identified (Huang et al., 2007). Taken together, our understanding of ABA-perception, ABA-signalling networks and whole plant ABA-mediated plant responses is still very fragmentary and incomplete. Further elucidation of the particularly complex mechanisms of responses to ABA will help to clarify many important plant processes in which ABA is involved, including the response to pathogens.

5. ABA HAS A PROMINENT ROLE IN INTEGRATING STRESS SIGNALLING NETWORKS

When responding to different stresses, integration of stress signalling networks is essential for an adequate response of appropriate amplitude. Economical use of biochemical resources implies a significant amount of overlap between the responses to different stresses and explains the use of common signalling components in the response to both biotic and abiotic stresses. Furthermore, biotic and abiotic stress responses can even show considerable overlap at the level of signal perception. For example, root rot or wilting pathogens can cause dehydration stress and thereby trigger an abiotic stress response. On the other hand, different types of stress require distinct and specific responses. The need to prioritize specific stress responses coupled to simultaneous down-regulation of others, justifies the antagonistic interplay commonly observed between different stress signalling networks.

5.1. ABA concentrations mediate global shifts in stress response priority

In nature, the co-occurrence of drought stress and pathogen attack is rare, as the great majority of pathogens require relatively humid conditions for infection and the establishment of disease (Agrios, 2005). Furthermore, drought or dehydration stress forms a much greater threat to plant survival than pathogen infection, which is consistent with the plant's need to be able to quickly prioritise drought stress responses at the expense of growth and the responses to other stresses. ABA-responsive signalling functions as a global switch to activate the drought stress response and represses many other plant processes, among those the response to pathogens. This is consistent with the findings of Anderson et al. (2004) who showed that JA/ET-dependent defence gene suppression by ABA cannot be reversed by JA or ET application, demonstrating that ABA action is a dominant process. The strong antagonistic effect between abiotic and pathogen responses is also exemplified in the ABA-deficient *sitiens* tomato mutant. When grown under conditions of high relative humidity, ABA deficiency does not result in major morphological abnormalities. Nevertheless, ABA-deficient plants are unable to cope with drought or cold stress due to the lack of ABA-mediated stomatal regulation (Nagel et al., 1994). However, the ability of ABA-deficient tomato to block the necrotrophic pathogens *B. cinerea* and *Erwinia chrysanthemi* reveals its enormous defensive capacity towards biotic stress (Asselbergh et al., 2007; Asselbergh et al., 2008; see chapters 2 and 5). This was also reflected

at the transcriptome level, as *sitiens* exhibits higher expression of defence-related genes prior to infection and shows a further elevation quickly after *B. cinerea* inoculation (Asselbergh et al., 2007). This demonstrates that deficiency in ABA results in a global shift towards strong pathogen defence responses at the expense of reduced tolerance to abiotic stress. Taken together with the strong negative effects of ABA on disease phenotypes and the function of ABA as a virulence factor (see paragraphs 2.2 and 2.3) against the central role of ABA in abiotic stress responses, it seems that in general strong decreases in ABA levels lead to hyperactivation of pathogen defence together with a reduced capacity to react to abiotic stress, whereas elevation of ABA levels lead to enhanced abiotic stress responses and suppression of pathogen defences responses.

5.2. ABA integrates and fine-tunes different stress responses

In addition to the apparent role of ABA levels in mediating a global shift between abiotic and biotic stress responses, ABA-responsive signalling seems to interfere at multiple steps in various signal transduction cascades, leading to fine-tuning and integration of different stress responses. As discussed, ABA influences disease signalling both positively and negatively. An overview of ABA action on pathogen defence responses described in this paper is given in figure I-1. Additional points of convergence between the signalling responses to abiotic and biotic stress have been characterised (reviewed by Fujita et al., 2006; Mauch-Mani and Mauch, 2005). However it seems that only a tiny portion of the total overlap of abiotic and biotic stress signalling networks has been described. Several signalling mechanisms of high complexity are shared between ABA abiotic stress signalling and pathogen defence and constitute means of overlap between different pathways, including Ca^{2+} and Ca-dependent protein kinase signalling (Klüsner et al., 2002; Ludwig et al., 2004), ROS- and nitric oxide-signalling (Pastori and Foyer, 2002; Apel and Hirt, 2004), mitogen-activated protein kinase (MAPK) signalling cascades (Xiong and Yang, 2003; Fujita et al., 2006) and various transcription factor families, containing functional domains such as AP2, WRKY, bZIP/HD-ZIP, MYB, MYC and several classes of zinc-fingers (Chen et al., 2002; Li et al., 2004; Zhu et al., 2005; Andersen et al., 2004; Mengiste et al., 2003).

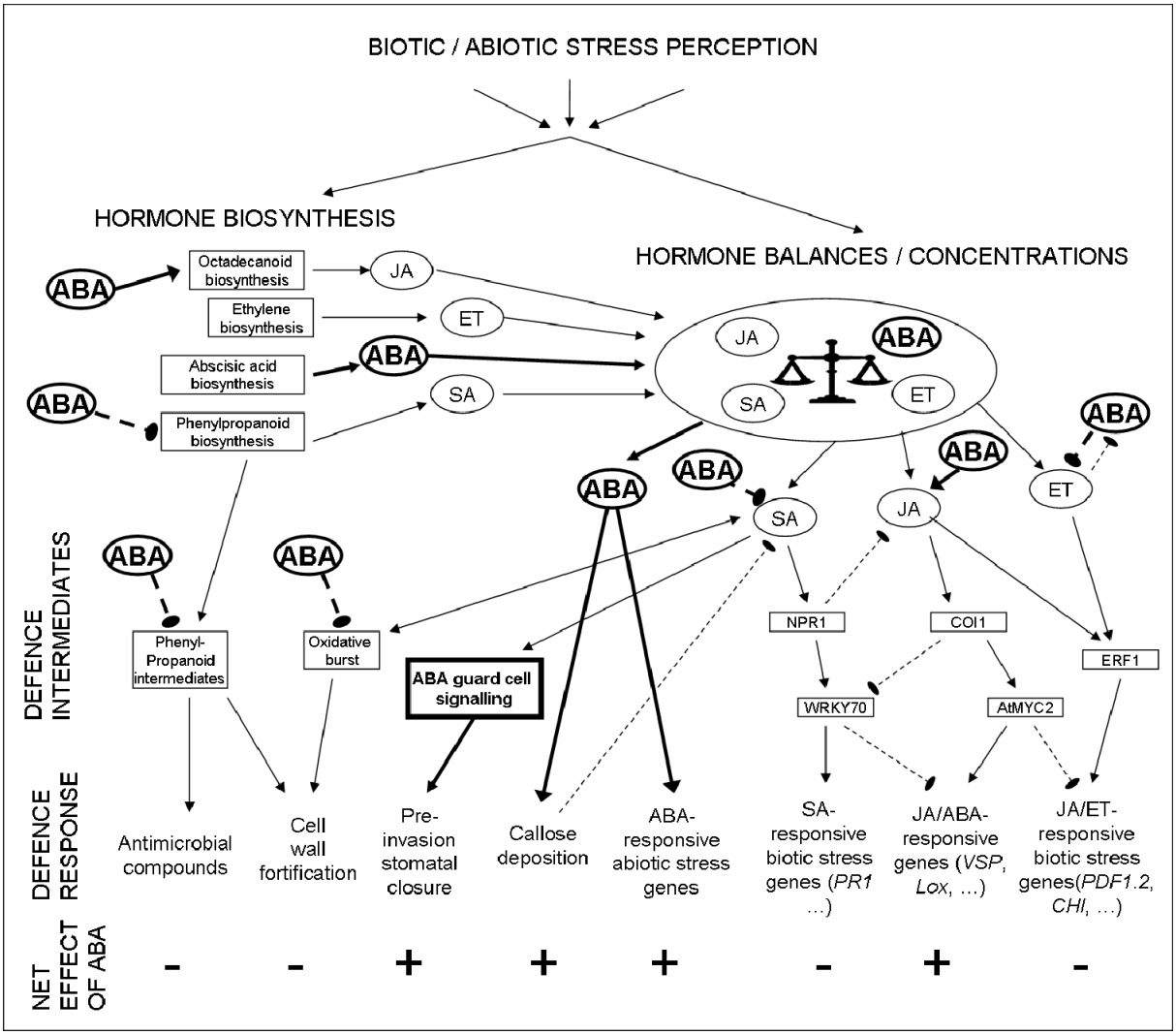

Figure I-1. Schematic representation of ABA interfering with plant abiotic and biotic stress responses. Emphasis is given to ABA influencing pathogen defence responses via different modes of action and not to the functions of ABA signalling in abiotic stress responses. The representation of JA/ET/SA cross-talk involving the transcription factors AtMYC2, NPR1, COI1, ERF1, and WRKY70 was partly based on a model by Lorenzo and Solano (2005). Sharp full-line arrows represent stimulatory effects and blunt dotted line arrows represent repressive effects. Effects of ABA are marked in bold.

The number of reports that functionally characterise transcription factors and signalling components involved in both biotic and abiotic stresses is growing fast and originates from studies on various plant species. For example, the pepper C3-H-C4 type RING-finger protein CaRFP1 functions as an early defence regulator controlling disease susceptibility and osmotic stress tolerance, probably by influencing SA and ABA signalling respectively (Hong et al., 2007). Also, the barley ERF-type transcription factor HvRAF enhances pathogen resistance and salt tolerance (Jung et al., 2007). Ectopic expression of HvRAF in Arabidopsis confers its conserved function. Similarly, ectopic expression of the rice Osmyb4 upstream transcription factor in Arabidopsis was shown to have a positive effect on the responses to abiotic (cold, drought, salt), environmental (ozone, UV) and biotic (TNV, *B. cinerea*, *P. syringae*) stresses

(Vannini et al., 2006). However, overexpression of the same gene in tomato only improved tolerance to drought stress and virus infection, while other stress responses were not improved (Vannini et al., 2007), demonstrating that the conservation of stress response machinery in dicotylous plants is only partial. Further unravelling of the components regulating the signalling events between different stress stimuli and their resulting defence measures will contribute in understanding the integration of overlapping stress signalling networks and the complex role of ABA herein. Even greater challenges will presumably lie in combining information from different plant species on partially conserved stress response signalling networks and transferring this knowledge in applied agricultural benefits.

ACKNOWLEDGEMENTS

This work was supported by the "Fonds voor Wetenschappelijk Onderzoek-Vlaanderen" (grants no. G.0350.04 and G.0061.03) and "Bijzonder Onderzoeksfonds" of the Ghent University.

Chapter

2

Resistance to *Botrytis cinerea* in *sitiens*, an Abscisic Acid-Deficient Tomato Mutant, Involves a Timely Production of Hydrogen Peroxide and Cell Wall Modifications in the Epidermis

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Plant defence mechanisms against necrotrophic pathogens such as *Botrytis cinerea* are considered to be complex and to differ from those that are effective against biotrophs. In the ABA-deficient *sitiens* tomato (*Solanum lycopersicum)* mutant, which is highly resistant to *B. cinerea*, accumulation of hydrogen peroxide was earlier and stronger than in the susceptible wild type at the site of infection. In *sitiens*, hydrogen peroxide accumulation was observed from 4 h post inoculation, specifically in the leaf epidermal cell walls, where it caused modification by protein cross-linking and incorporation of phenolic compounds. In wild-type tomato plants, hydrogen peroxide started to accumulate 24 h post inoculation in the mesophyll layer and was associated with spreading cell death. Transcript profiling analysis using TOM1 microarrays revealed that defence-related transcript accumulation prior to infection was higher in *sitiens* than in wild type. Moreover, further elevation of *sitiens* defence gene expression was stronger than in wild type 8 h post inoculation, both in number of genes and in their expression levels and confirmed a role for cell wall modification in the resistant reaction. Although in general, plant defence-related reactive oxygen species formation facilitates necrotrophic colonization, these data indicate that a timely hyperinduction of hydrogen peroxide-dependent defences in the epidermal cell wall can effectively block early development of *B. cinerea*.

INTRODUCTION

Botrytis cinerea causes grey mould diseases in a broad range of plant species and is one of the most comprehensively studied necrotrophic plant pathogens. Necrotrophs kill their host cells by secreting toxic compounds or lytic enzymes and in addition produce an array of pathogenicity factors that can subdue host defences (reviewed by van Kan, 2006). Despite elaborate research studies, the biochemical and genetic basis of resistance to *Botrytis* is still not fully understood. The ability of the fungus to kill cells was proposed as a major determinant in the host specificity of different *Botrytis* species (Mansfield and Hutson, 1980) and similarly, plant resistance to *Botrytis* is supposed to depend on the balance between cell death and survival (van Baarlen et al., 2007). In addition, constitutive and inducible plant secondary metabolites determine host specificity of different *Botrytis* spp. and fungal colonization in compatible interactions (reviewed in van Baarlen et al., 2004a). Structural barriers and cell wall fortifications are also considered to be involved in arresting *Botrytis*, although the actual contribution to the effective inhibition of infection is often unclear (van Baarlen et al., 2004a).

One of the most important plant defence responses to pathogens is the production of reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), during the oxidative burst (Mehdy, 1994). ROS can function in cell wall modification, defence signalling, the hypersensitive response (HR) or be directly toxic to pathogens (Lamb and Dixon, 1997). There is evidence, however, that generation of ROS assists the colonization of plant tissue by necrotrophic pathogens: *B. cinerea* infection can be suppressed by spraying of antioxidants on plants (Elad, 1992) and H_2O_2 is produced during common bean (*Phaseolus vulgaris*) and *Arabidopsis thaliana* colonization (von Tiedemann, 1997; Govrin and Levine, 2000). Therefore, defence reactions effective against biotrophic pathogens are believed to increase vulnerability toward necrotrophs (reviewed by Lyon et al., 2004). Nevertheless, the role of ROS in defence against *B. cinerea* remains controversial. Some studies suggest a positive effect of ROS on plant resistance: chemical induction of an oxidative burst in tomato with o-hydroxyethylorutin resulted in resistance (Malolepsza and Urbanek, 2002) and a biphasic oxidative burst has been reported in bean cells attacked by *B. cinerea* (Unger et al., 2005). The secondary oxidative burst was much stronger in infections with a non-aggressive than with an aggressive strain, which led to the conclusion that ROS-mediated HR-like cell death was able to block *B. cinerea*.

Four loci (*BOS1*, *BOS2*, *BOS3*, and *BOS4*) that affect Arabidopsis susceptibility to *B. cinerea* were recently identified. The *BOS1* locus has been shown to encode a MYB

transcription factor that interacts with the jasmonate signalling pathway mediated by ROS (Mengiste et al., 2003; Veronese et al., 2004). Within the complex transcriptional response of Arabidopsis during infection, subsets of the *Botrytis*-induced genes depend on functional jasmonate, ethylene, and salicylic acid (SA) signalling pathways and three *Botrytis*-induced transcription factors are sensitive to ABA (AbuQamar et al., 2006). These results demonstrate that the outcome of interactions between plants and necrotrophs is -as with biotrophs- determined by a complex interplay between different plant hormones regulating defence gene expression and disease resistance (Thomma et al., 1998; Glazebrook, 2005).

Recently, it has become clear that there is an important overlap between biotic and abiotic stress signalling involving ABA (Xiong and Yang, 2003; Anderson et al., 2004; Fujita et al., 2006). Some exceptions notwithstanding, high or basal ABA levels contribute to a susceptible response to the pathogen (Henfling et al., 1980; Mohr and Cahill, 2001; Mauch-Mani and Mauch, 2005; see chapter 1). During the plant's defence to pathogens, interactions of ABA with SA, jasmonate or ethylene are mostly antagonistic, as is the case in several plant developmental processes. However, besides the interaction of ABA with other hormones in defence signalling, there is little or no knowledge on the primary mechanisms of ABA-induced disease susceptibility (Mauch-Mani and Mauch, 2005). During abiotic stress, ABA-derived signal transduction often involves accumulation of H_2O_2 (Kwak et al., 2006). On the other hand, in physiological processes such as seed germination, the release of H_2O_2 is inhibited by ABA (Schopfer et al., 2001).

The tomato ABA-deficient *sitiens* mutant is highly resistant to *B. cinerea* and displays a stronger SA-dependent defence response than the wild type (Audenaert et al., 2002a). In addition, it is less susceptible to the biotrophic fungal pathogen *Oidium neolycopersici* (Achuo et al., 2006), the hemi-biotrophic bacterium *Pseudomonas syringae* pv. *tomato* (Thaler and Bostock, 2004) and the necrotrophs *Sclerotinia sclerotiorum* and *Erwinia chrysanthemi* (Asselbergh et al., 2008; see chapter 5). Here, we show that a rapid H_2O_2 accumulation is essential within the resistance mechanism of *sitiens* to *B. cinerea*. H_2O_2 accumulated rapidly in the *sitiens* epidermal cells upon pathogen inoculation, causing increased protein cross-linking and peroxidative incorporation of phenolic compounds in the cell wall; reactions that were not present in the wild type. This fast cell wall-related defence response was also reflected at the transcriptome level with increased defence-related transcript levels. Our data suggest that timing, quantity, and localization of ROS determine the outcome of the *B. cinerea*-tomato interaction.

RESULTS

Botrytis cinerea is blocked in the early steps of the infection process in *sitiens*

The first symptoms on tomato leaf tissue inoculated with a *B. cinerea* conidial suspension appear as necrotic spots beneath the inoculation droplet between 24 and 48 h post inoculation (hpi). In a susceptible reaction, these primary necrotic spots develop to water-soaked, macerated lesions within 96 hpi (Benito et al., 1998). In a resistant reaction, fungal development is restricted to a few dark-brown spots (non-spreading lesions). After conidia inoculation, the ABA-deficient *sitiens* mutant shows primary necrotic spots, but displays a strong reduction in the percentage of spreading lesions compared to wild-type plants (Audenaert et al., 2002a). In the current study, we specifically assessed the differences between wild type and *sitiens* during the early stages of the infection process. We modified the drop inoculation method described by Audenaert et al. (2002a) by adding a "pre-germination" step to synchronize *B. cinerea* conidia germination and by using 1 cm diameter leaf discs floating on water. This procedure leads to a more uniform and synchronized infection, without compromising the resistance response in *sitiens* (Fig. II-1A).

Until 24 hpi, infection events were very similar in wild-type and *sitiens* leaf discs. Conidial attachment and germination occurred within 4 hpi, followed by normal hyphal growth, accompanied with appressorium-mediated and hyphal tip penetration attempts (data not shown). The first differences were observed during primary necrosis events between 24 and 48 hpi (Fig. II-1B): in *sitiens*, spots were more abundant and appeared as dry, dark-brown, round-shaped dots, whereas in wild-type leaf tissue, necrotic spots were larger, irregularly shaped and pale-brown. These observations suggest that in *sitiens*, resistance mechanisms are already activated prior to −or at latest during− the onset of primary necrosis.

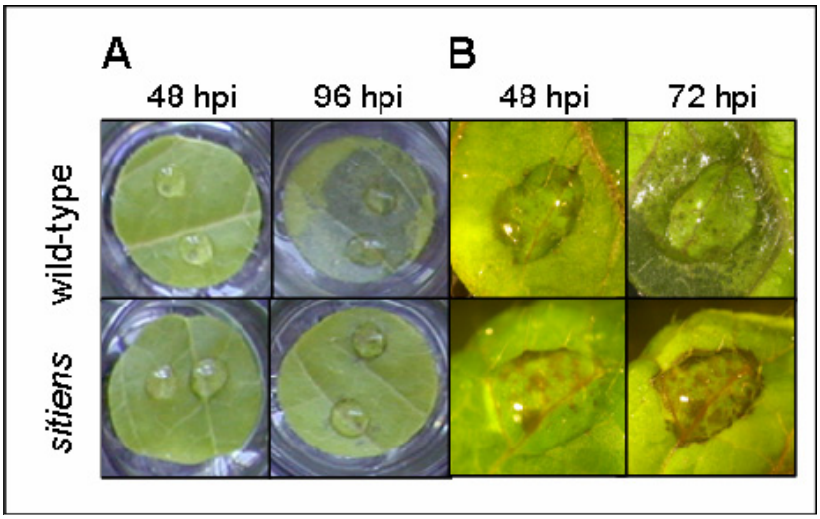

Figure II-1: Disease symptoms on wild-type and *sitiens* tomato leaf discs after inoculation with two 5-µl drops of a *B. cinerea* conidial suspension.
A: Macroscopic disease symptoms. In the wild-type leaf discs, spreading grey disintegration of the host tissue is visible and in *sitiens* discs necrotic spots at the site of the inoculation droplet are observed. B: Close up of disease symptoms. In *sitiens*, primary necrotic lesions contain a high number of distinct dark spots at 48 and 72 hpi. In wild type, primary necrotic spots at 48 hpi are typically larger, less numerous, have a paler shade of brown and more often develop into spreading water-soaked lesions.

Resistance in *sitiens* is associated with a rapid accumulation of H_2O_2 in epidermal cells

Because ROS production is one of the earliest defence responses in plant-pathogen interactions, we compared H_2O_2 accumulation at the inoculation site by using 3,3'-diaminobenzidine (DAB) staining. In this protocol, brown precipitates are formed at the sites of H_2O_2 accumulation (Thordal-Christensen et al., 1997). Wild-type and *sitiens* leaf discs were inoculated with a droplet of a *B. cinerea* conidial suspension and floated for 3 h on a solution containing 1 mg/ml DAB before sampling 4, 8, 12, 24, 48 and 72 hpi. In mock-inoculated leaf discs, no DAB accumulation was observed. In wild type, DAB staining was macroscopically detectable at 48 hpi and was associated with lesion progression. In *sitiens*, staining became already macroscopically visible from 8 hpi and further intensified at later time points, but remained restricted to the area covered by the inoculation droplet (Fig. II-2). When the DAB solution was supplemented with ascorbic acid, staining was abolished, indicating that the staining was due to H_2O_2 accumulation (data not shown).

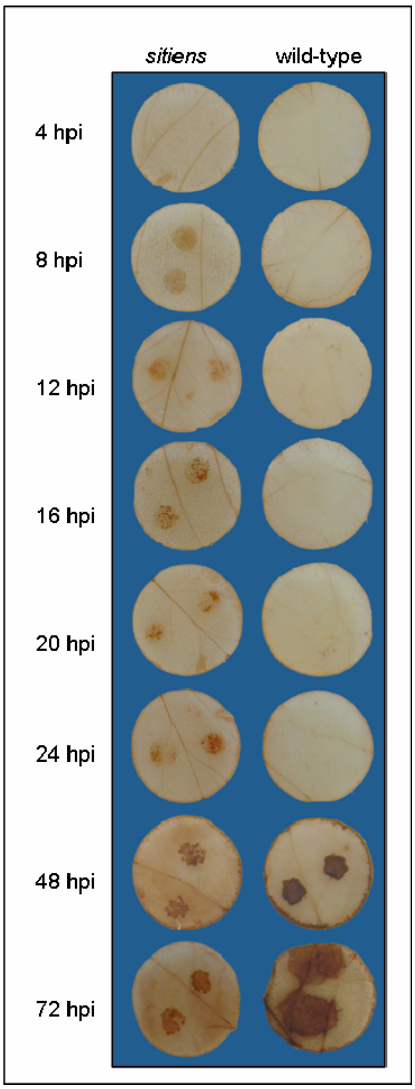

Figure II-2: Temporal evolution of H_2O_2 accumulation in wild-type and *sitiens* tomato after infection with *B. cinerea*.
DAB staining of leaf discs infected with two 5-µl drops of a conidial suspension was performed at different time points post inoculation (4, 8, 12, 16, 20, 24, 48, and 72 hpi). One representative disc out of three replicates is shown for each time point.

Microscopic observations revealed that in *sitiens* DAB staining was detectable as early as 4 hpi in epidermal cell walls in close contact to the fungal germ tubes (Fig. II-3A). Between 4 and 8 hpi, H_2O_2 accumulation was detected also in the entire anticlinal wall of epidermal cells in contact with the fungus and in neighbouring epidermal cells. The spreading of H_2O_2 accumulation from the sites of fungal contact resulted in a general DAB staining of mainly the anticlinal walls of the majority of epidermal cells beneath the inoculation droplet at 8 hpi (Fig. II-3B). At later time points, extracellular H_2O_2 accumulation gradually decreased, but from 12 hpi, H_2O_2 was also clearly visible inside multiple epidermal cells in *sitiens* (Fig. II-3C). Epidermal cells with intracellular H_2O_2 accumulation displayed autofluorescence, due to accumulation of phenolic compounds, and cytoplasmic aggregation (Fig. II-4), two features that are considered as hallmarks of a HR (Heath, 2000). None of these reactions were visible in wild-type cells at these early time points. Instead, the developing lesion showed an intense DAB staining in the mesophyll layer that started between 24 and 48 hpi (Fig. II-3 and data not shown).

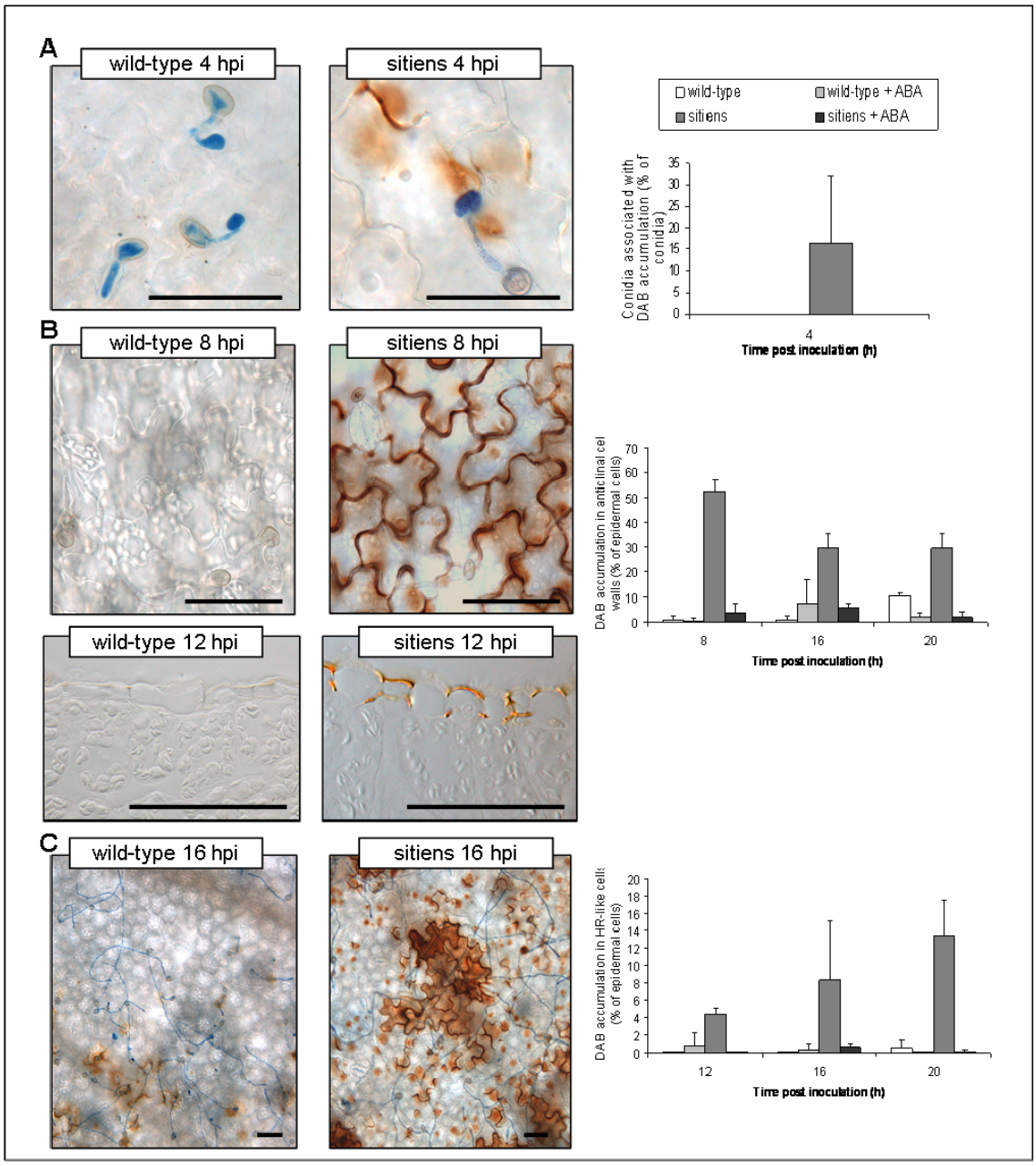

Figure II-3: Effect of ABA on H_2O_2 accumulation in epidermal cells after inoculation with *B. cinerea*.
A: Association of DAB accumulation with *B. cinerea* conidia. In *sitiens*, H_2O_2 was located at the site of penetration and in some parts of the anticlinal wall of the penetrated cell, whereas in wild type and in *sitiens* supplemented with ABA, no H_2O_2 accumulation was detected. Germinating conidia were classified in two groups based on the presence or absence of associated DAB accumulation in the epidermal cells, whose percentage is shown. B, DAB accumulation in epidermal cell walls. In *sitiens*, DAB staining was general in the entire outline of the anticlinal cell wall, whereas in wild type and *sitiens* supplemented with ABA, no H_2O_2 accumulation was detected at 8 hpi (upper panels). The restriction of *sitiens* DAB accumulation to the epidermal layer was confirmed on cross-sections (lower panels). Epidermal cells were classified in two groups based on the presence or absence of DAB accumulation in the anticlinal walls, whose percentage is shown in the anticlinal walls at 8, 16, and 20 hpi. C: Intracellular DAB accumulation in epidermal cells showing an HR-like reaction. At 16 hpi, wild-type and ABA-supplemented *sitiens* epidermal cells did not accumulate intracellular DAB, while in *sitiens*, groups of HR-like cells with intracellular DAB accumulation were present near the site of fungal penetration. Epidermal cells were classified in two groups based on the presence or absence of intracellular DAB accumulation and the percentage is shown at 12, 16, and 20 hpi. In all graphs, bars represent the means and the standard deviations of data from six inoculation droplets originating from three plants. In each inoculation droplet, at least 50 conidia (A) or 300 epidermal cells (B, C) originating from representative zones within each inoculation droplet were counted. The data of one experiment is presented. The experiment was repeated with similar results. Scale bar = 50 µm.

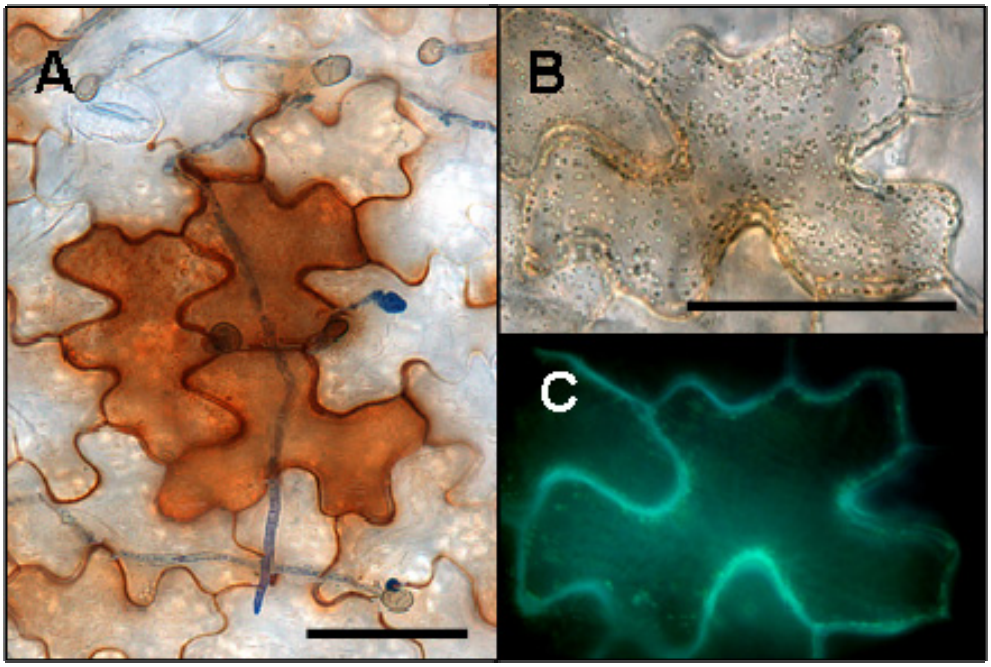

Figure II-4: HR-like reaction of *sitiens* epidermal cells at 12 hpi. Cells near the site of fungal penetration with this reaction showed intracellular DAB accumulation (A), cytoplasmic aggregation (B) and green to yellow autofluorescence after UV excitation (C). Scale bar = 50 µm.

Nitro blue tetrazolium (NBT) was used to visualize accumulation of superoxide (Doke, 1983) in wild-type and *sitiens* leaf tissue after inoculation with *B. cinerea*. NBT accumulated only at the leaf disc border and was not detected at the infection site at any of the examined time points (supplementary Fig. II-S1).

Hydrogen peroxide burst is necessary for *sitiens* resistance

To address whether the rapid H_2O_2 burst in *sitiens* is directly inferred from the ABA deficiency, exogenous ABA (100 µM) was applied before pathogen inoculation and DAB staining. Petiole feeding with exogenous ABA 24 h before inoculation was shown to restore *B. cinerea* susceptibility of *sitiens* to wild-type levels (Audenaert et al., 2002a). Repetitive spraying of ABA had the same effect (data not shown; see also Achuo et al., 2006) and suppressed H_2O_2 accumulation (Fig. II-5A). Microscopic assessment during the early stages of infection confirmed that ABA supplementation decreased the number of DAB-positive epidermal cells toward the amount detectable in wild-type tissue (Fig. II-3).

Artificial impairment of the rapid oxidative burst in *sitiens* also resulted in restoration of susceptibility. Floating of *sitiens* leaf discs on a solution supplemented with 1100 U/ml catalase or 5 mM ascorbate during conidia inoculation, significantly increased or totally re-established the susceptibility, respectively (Fig. II-5C). Whereas catalase treatment did not significantly affect susceptibility of the wild type, ascorbate provoked pathogen lesions to spread earlier than

in untreated leaf discs and led to abundant superficial fungal growth and accelerated sporulation (Fig. II-5C). Interestingly, only treatment with ascorbate completely eliminated all detectable H_2O_2 from the *sitiens* leaf tissue. The partial nature of the enzymatic H_2O_2 removal with catalase was observed both macroscopically (Fig. II-5A) and microscopically: catalase treatment resulted in a faint DAB staining that covered only part of the epidermal anticlinal walls (Fig. II-5B) without altering the number of cells that displayed extracellular or intracellular H_2O_2 accumulation (data not shown). Application of 50 μM diphenilene iodonium (DPI), an inhibitor of the ROS generating enzyme NADPH oxidase, restored *B. cinerea* susceptibility to wild-type levels (Fig. II-5C). DPI inhibited the specific accumulation of H_2O_2 underneath the inoculation droplet, but also caused a non-specific background staining covering the entire leaf disc surface (Fig. II-5A and 5B).

H_2O_2 accumulation in *sitiens* coincides with elevated levels of extracellular peroxidase and modification of the epidermal anticlinal cell walls

Because H_2O_2 production after pathogen attack can result from increased peroxidase activity and peroxidases mediate many H_2O_2-related defence responses, we examined extracellular peroxidase activity with the tetramethylbenzoidine (TMB) assay described by Ros Barceló (1998). Leaf discs inoculated with *B. cinerea* were fixed in ethanol and incubated in a solution of TMB and H_2O_2. Peroxidation of the TMB molecule resulted in a blue discoloration of both leaf tissue and incubation solution. The latter was used to quantify the activity of extracellular peroxidases (Lucena et al., 2003). In wild-type leaf discs, a minor increase was detected 72 hpi, while in *sitiens* peroxidase activity increased significantly between 4 and 24 hpi, followed by a drastic and sustained increase at 24 hpi (Fig. II-6). In mock-inoculated *sitiens* leaf discs, peroxidase levels also increased at 48 and 72 hpi, but levels remained lower than in infected *sitiens* discs.

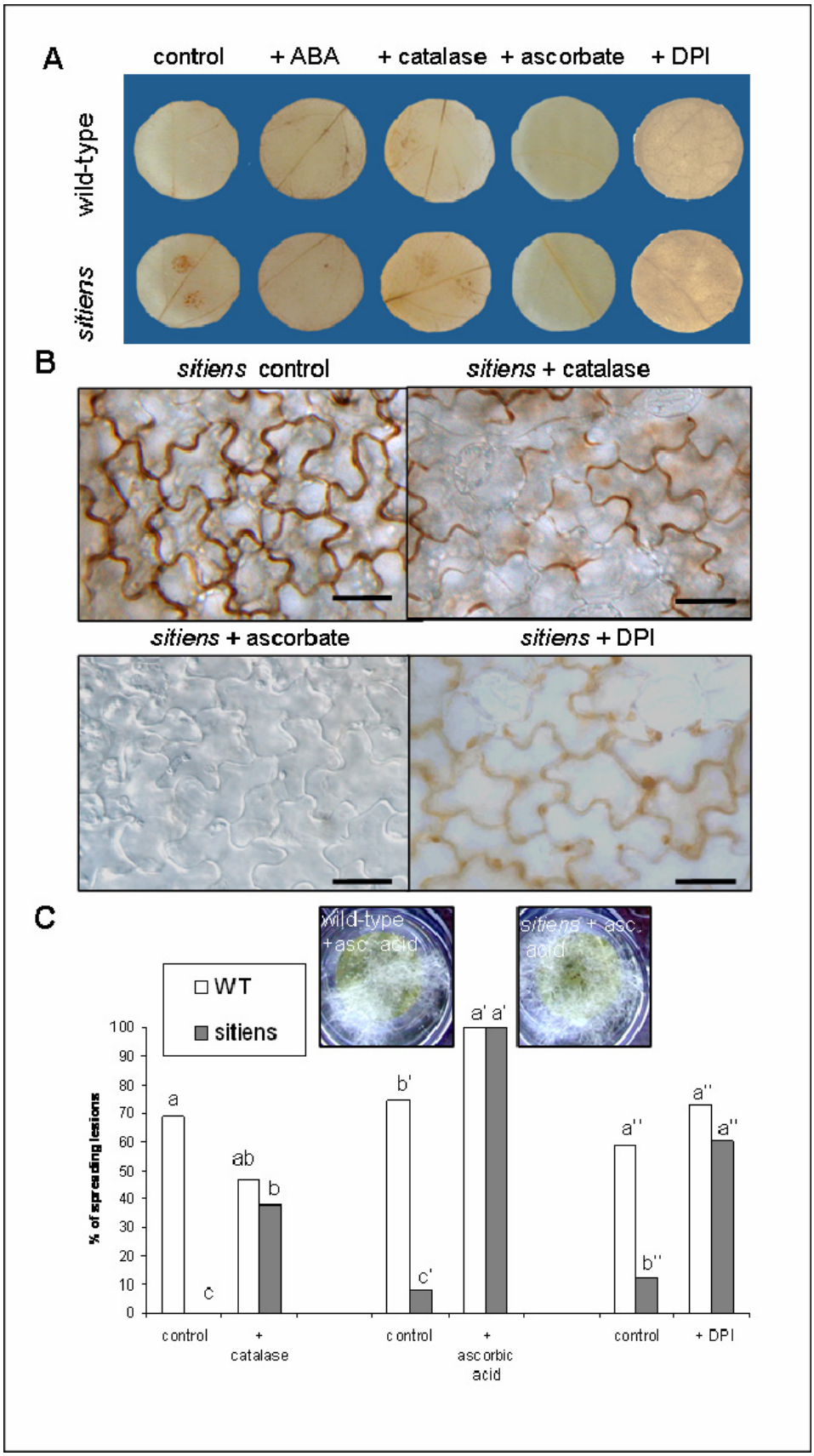

Figure II-5: Effect of ABA, ascorbate, catalase and DPI treatment on H_2O_2 accumulation and symptom development in wild-type and *sitiens* tomato leaf discs after inoculation with two 5-µl droplets of *B. cinerea* conidial suspension.
A: DAB staining at 20 hpi. One representative disc out of three replicates is presented. B: Micrograph of *sitiens* H_2O_2 accumulation at 8 hpi. C: *B. cinerea* symptom development. For each treatment at least 18 leaf discs from at least five plants were infected and the number of spreading lesions was evaluated at 4 dpi. The data were statistically analyzed using binary logistic regression. Bars with different letters are significantly different at $p<0.05$. Profuse pathogen development is visible on discs treated with ascorbic acid at 4 dpi. All experiments were repeated with similar results. Scale bar = 50 µm

Because peroxidase-dependent defence responses often use H_2O_2 as a substrate to cross-link cell wall components, we performed different staining procedures to visualise changes in

the cell wall. Cross-linking of cell wall proteins was detected with Coomassie blue subsequent to protein denaturation and free protein removal (Mellersh et al., 2002) (Fig. II-7A). In addition, we used safranin-o and toluidine blue to detect the peroxidative incorporation of phenolic compounds in the cell wall, a fortification mechanism important during lignification and suberization (Mellersh et al., 2002; Lucena et al., 2003) (Fig. II-7B and 7C). For both genotypes, no staining was visible outside the inoculation droplets. Cell wall modification was more abundant and appeared earlier in *sitiens* than in the wild type. Moreover, accumulation of all three stains clearly coincided in timing and location with the presence of extracellular H_2O_2: starting from 8 hpi, the anticlinal cell walls of *sitiens* epidermal cells stained intensely during the time course (12, 16, 20, 24, 48 and 72 hpi; data not shown), whereas in wild-type tissue, only limited zones of the anticlinal cell walls of a few cells were positive and only after 12 hpi (Fig. II-7). Underneath the inoculation droplet, the number of epidermal cells displaying clear cell wall modification in the anticlinal walls ranged between 40% and 80% in *sitiens*, but was never higher than 20% in wild-type cell walls (data not shown).

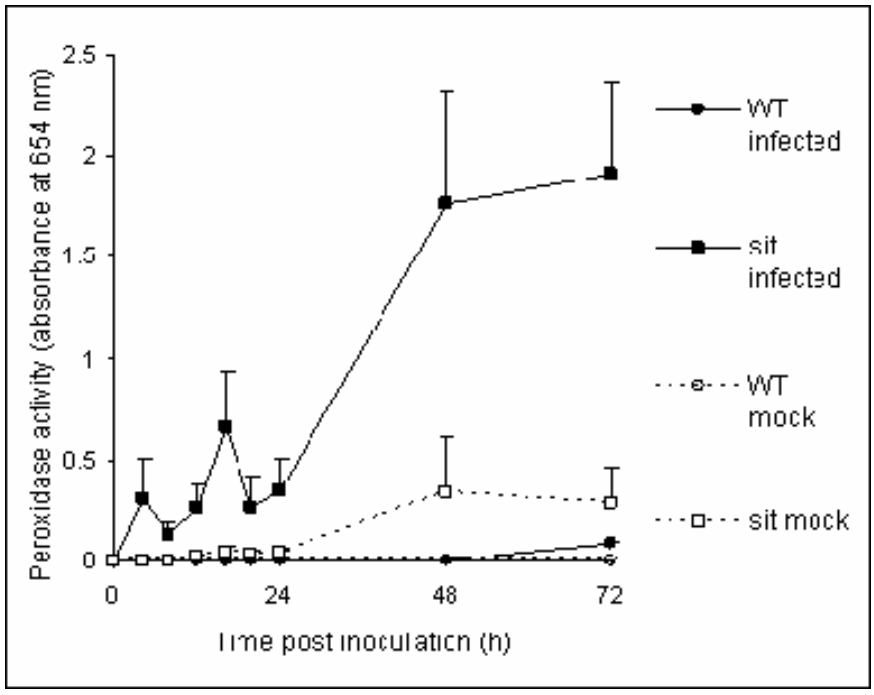

Figure II-6: Extracellular peroxidase activity in wild-type and *sitiens* leaf discs infected with *B. cinerea*.
Leaf discs were inoculated with two 5-µl droplets of *B. cinerea* (infected) or with the control (mock) solution and fixed in ethanol at the different time points. Peroxidase activity was measured at 654 nm after addition of TMB and 0.03% H_2O_2. The mean and standard error of the absorbance of the incubation solution from three discs of different plants are presented.

H_2O_2 dependency of cell wall modification in *sitiens* was further confirmed on leaf discs treated with antioxidants. While ascorbate treatment removed all *sitiens* cell wall modifications, treatment with catalase resulted in a lower staining intensity (Fig. II-8).

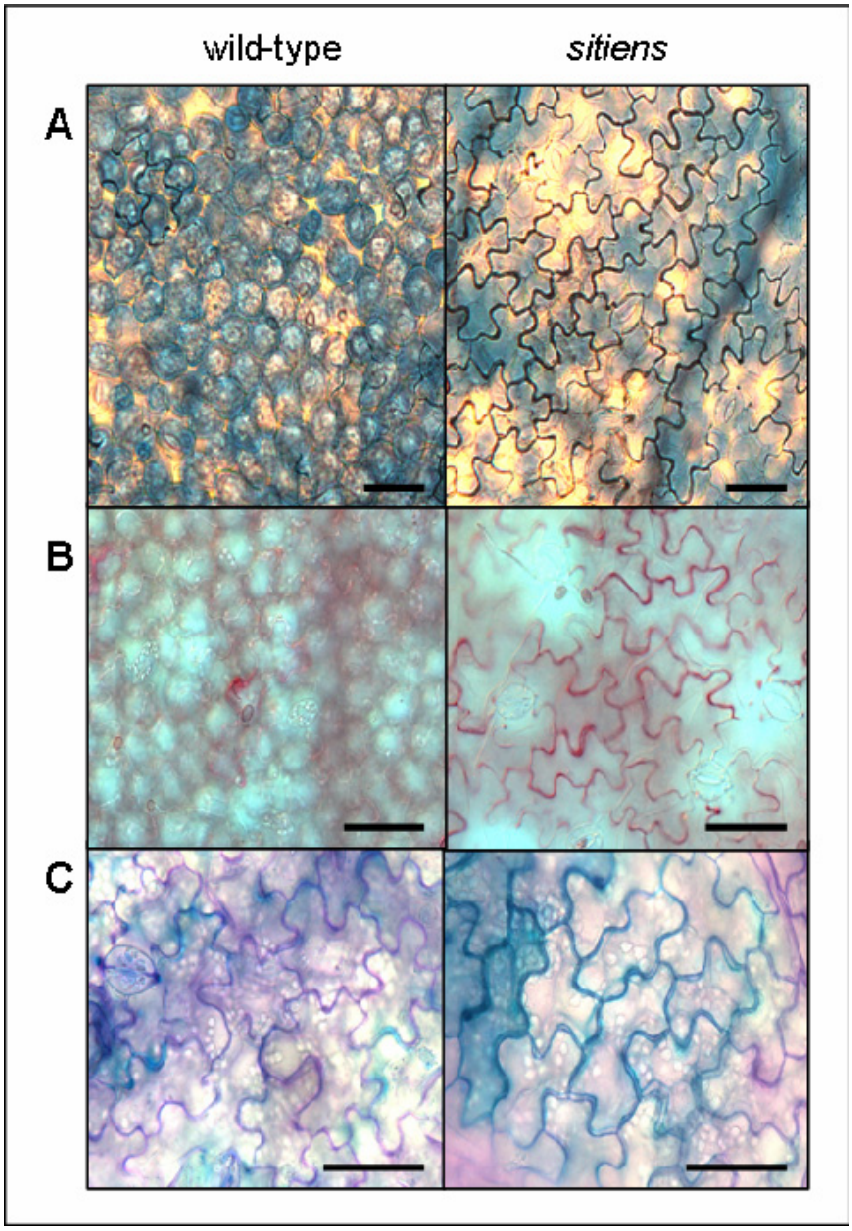

Figure II-7: Cell wall modifications in wild-type and *sitiens* tomato inoculated with *B. cinerea*.
In *sitiens*, cell wall modifications were first detected at the sites of H_2O_2 accumulation and were generally present from 8 hpi beneath the inoculation droplet in the epidermal anticlinal cell walls, with an increase in staining intensity at subsequent time points. In wild-type tomato, only few cells had limited cell wall modifications. Cell wall modifications were visualised with Coomassie blue staining after SDS denaturation to detect protein cross-linking (dark blue) (A); with safranin-o (red-pink) (B); and with toluidine blue to detect phenolic compounds (turquoise) whereas pectic fragments stain purple (C). For each time point and stain, infection sites on at least 3 leaf discs from independent *sitiens* and wild-type plants were examined and gave the same pattern of cell wall fortification. Scale bar = 50 µm.

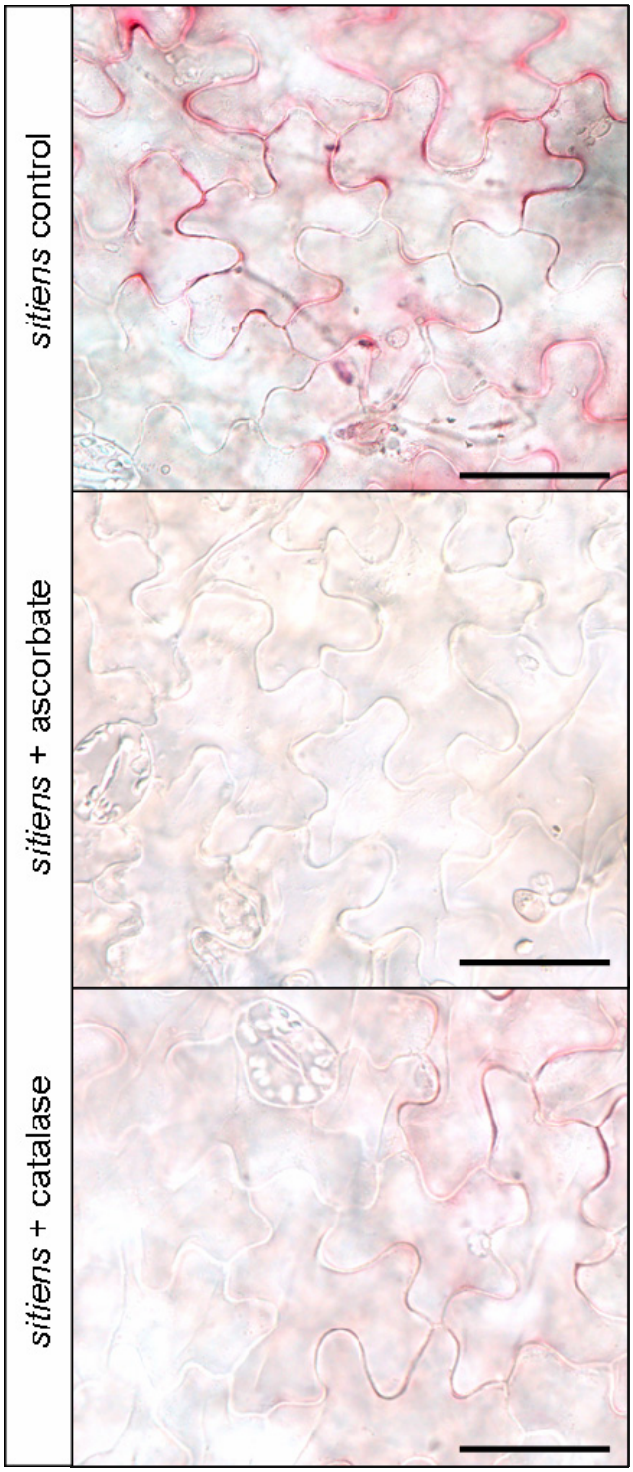

Figure II-8: Effect of ascorbate and catalase treatment on epidermal cell wall modification in *sitiens* tomato inoculated with *B. cinerea*.
Sitiens leaf discs that were floated in a solution of ascorbate or catalase and infected with *B. cinerea* were fixed in ethanol at 16 hpi and stained with safranin-o. Ascorbate treatment completely removed the cell wall staining, whereas catalase treatment resulted in a fainter accumulation of the stain compared to the control treatment.
For each treatment, at least 3 discs were examined and gave the same pattern of cell wall modification. Scale bar = 50 µm.

Transcriptome profiling of the tomato response to *B. cinerea*

To assess changes in gene expression, we compared the transcriptome of wild-type and *sitiens* plants inoculated with *B. cinerea*. Detached leaves were spray-inoculated with a conidial suspension to ensure a standardised sampling procedure because of a uniform infection covering the entire leaf area. Disease scoring showed that symptom development was similar to

that after drop inoculation, with *sitiens* being more resistant than the wild type (Supplementary Fig. II-S2). We performed a genome-wide transcriptome analysis with TOM1 tomato cDNA arrays containing 12 899 expressed sequence tags (ESTs), representing approximately 8600 unigenes (Alba et al., 2004). A loop design was constructed, consisting of seven replicated dye-swap experiments in which two independent pools of four leaflets harvested from 5-week-old mock-sprayed and spray-inoculated plants at 0 and 8 hpi were compared in an equal treatment replication structure, on a total of 14 TOM1 cDNA microarrays (Supplementary Fig. II-S3). We chose to sample at 8 hpi because at that stage no necrosis is visible yet, despite obvious fungal penetration attempts and H_2O_2 accumulation. In addition, time course analyses with cDNA-amplified fragment length polymorphism indicated that gene expression induced by *B. cinerea* was first detectable between 4 and 8 hpi (data not shown). Similar to previous studies (Vuylsteke et al., 2005), we used a mixed-model approach to produce estimates of the gene-specific genotype, treatment and genotype×treatment effects along with appropriate standard errors. Briefly, a linear mixed normalization model was used to estimate the global variation of the collection of the 12 899 cDNA fragments in the form of random array effects, random channel effects, and random error. In a second step, 12 899 gene-specific models were applied to partition the gene-specific variation into fixed dye effects, fixed treatment (e.g. genotype, inoculation, time) effects, random spot effects, and random error. In a third and last step, for each transcript, expression differences were estimated for contrasts among treatments and were tested by t-tests. In order to make tables within the paper not too exhaustive, we used a conservative *p*-value cut-off of 0.001, whereas results with a cut-off of 0.01 are given as supplementary data (Table II-SI). Microarray results were confirmed with quantitative real-time (qRT-)PCR with gene-specific primers (see "Materials and Methods") for six genes selected based on following analyses (Supplementary Fig. II-S4).

First, we assessed differential gene expression between wild type and *sitiens* prior to pathogen inoculation. Differential expression ($p<0.001$ and >2 fold change (FC)) was evident for 56 ESTs representing 40 unigenes (Table II-I). Within the unigenes upregulated in *sitiens* compared to wild type (28), there was a clear enrichment for proteins involved in defence responses. The strongest upregulated genes (>5 FC) were almost all associated with plant-pathogen interactions: pathogenesis-related protein PR1, osmotin-like proteins, glucan endo-1,3-β-glucosidase B, and thaumatin-like proteins (van Loon and van Strien, 1999), suggesting that *sitiens* plants are already in a "pre-alerted" state of defence before pathogen infection. Interestingly, the expression of the pathogenesis-related transcriptional activator Pti5

was also higher in *sitiens* prior to inoculation.

Table II-I: Non-redundant list of genes significantly differentially expressed in wild-type and *sitiens* plants prior to inoculation with *B. cinerea* (p<0.001).

Unigene ID	Gene description	ESTs	FC	GO annotation
Genes with higher expression in *sitiens* than in wild type				
SGN-U143838	Pathogenesis-related protein PR-1	4	11.14	Biotic stress
SGN-U144488	Osmotin-like protein (PA15)	1	10.67	Biotic stress
SGN-U150295	Expressed protein	1	9.64	No ontology
SGN-U143416	Glucan endo-1,3-β-glucosidase B	3	7.93	Misc. β 1,3 glucan hydrolases
SGN-U160528	Thaumatin homolog NP24	1	7.23	Biotic stress
SGN-U147967	No hits found	1	5.99	Unknown
SGN-U143414	NP24 protein (P23)	7	5.61	Biotic stress
SGN-U145000	Wound-induced protein	3	4.40	Abiotic stress
SGN-U143337	Endochitinase 3	1	3.97	Biotic stress
SGN-U149296	Protease inhibitor/seed storage/lipid transfer protein (LTP) family	1	3.54	Misc. protease inhibitor
SGN-U155388	Activator-like transposable element	1	3.34	DNA synthesis
SGN-U144273	Expressed protein	1	3.33	No ontology
SGN-U146585	Putative membrane protein	1	3.32	Hormone metabolism.
SGN-U145477	Peroxidase	1	2.98	Misc. peroxidases
SGN-U147667	Expressed protein	1	2.72	Unknown
SGN-U145711	Disease resistance response protein-related/dirigent protein-related	2	2.66	Biotic stress
SGN-U147854	Pathogenesis-related transcriptional activator Pti5	1	2.61	Hormone metabolism
SGN-U143283	Putative glutathione S-transferase	1	2.61	Misc. glutathione S-transferases
SGN-U148968	Expressed protein	1	2.54	Signalling
SGN-U145988	AAA-type ATPase family	2	2.49	Protein degradation
SGN-U146340	Nine-cis-epoxycarotenoid dioxygenase 4	1	2.37	Hormone metabolism
SGN-U143678	Actin	1	2.32	Cell. organisation
SGN-U144386	14-3-3 protein 7	1	2.14	Signalling
SGN-U151205	Expressed protein	1	2.12	Unknown
SGN-U146043	Putative pathogenesis-related protein	1	2.09	Biotic stress
SGN-U157113	No hits found	1	2.04	Unknown
SGN-U154156	Hypothetical protein	1	2.04	Unknown
SGN-U147469	Acetyl-CoA C-acyltransferase	1	2.01	Lipid metabolism
Genes with lower expression in *sitiens* than in wild type				
SGN-U143179	Lipid transfer protein 2	2	-3.47	Lipid metabolism
SGN-U144375	Monooxygenase	1	-3.19	Misc. oxidases
SGN-U151742	Small blue copper protein Bcp1	1	-3.01	Misc. plastocyanin like
SGN-U145371	Anthocyanidin 3-O-glucosyltransferase	1	-2.60	Misc. UDP glucosyl and glucoronyl transferases
SGN-U144440	B-amyrin synthase	1	-2.27	Secondary metabolism.isoprenoids
SGN-U144270	Probable cytochrome P450	1	-2.16	Misc. cytochrome P450
SGN-U143901	Cytochrome P450 family	1	-2.16	Misc. cytochrome P450
SGN-U148216	Receptor protein kinase-like protein	1	-2.15	Posttranslational modification
SGN-U144995	No hits found	1	-2.11	Unknown
SGN-U151022	Chlorophyllase	1	-2.03	Tetrapyrrole synthesis
SGN-U144679	Annexin -related	1	-2.02	Cell. organisation
SGN-U147643	Ca2+/H+-exchanging protein	1	-2.01	Transport

FC: Fold change of expression in *sitiens* compared to wild type. When more than one EST for the same gene is present in the data, the EST with the highest FC is presented.

Next, we filtered the genes that were significantly differentially expressed between mock-inoculated and pathogen-inoculated samples. In wild type, 11 genes were significantly differentially regulated 8 hpi upon pathogen inoculation (p<0.001), whereas in *sitiens* 41 were. In wild type, two extensins and a cell wall protein were significantly modulated by the *B. cinerea* inoculation with a FC of at least 1.5 compared to 26 unigenes in *sitiens* (Table II-II; Supplementary Table II-SI, p<0.01), indicating a stronger response to inoculation in *sitiens* than in wild type at 8 hpi. Most prominent inductions were observed for pathogen response-related genes, including PR-1A1, PR1, an extensin, a protease inhibitor, and a lipoxygenase. Because

of the involvement of cell wall-related defence mechanisms in *sitiens* resistance as indicated by the previous experiments, we assessed expression patterns of cell wall-related genes upon inoculation. Table II-SII presents the gene ontology-annotated cell wall genes together with five genes that we manually annotated as cell wall-related genes based on literature surveys, which were deregulated upon pathogen inoculation in *sitiens* with a fold change of at least 1.5. Most of these cell wall genes are involved in cross-linking, supporting the evidence for rapid cell wall modification in the resistance of *sitiens*.

Table II-II: Non-redundant list of genes significantly differentially expressed in wild-type or *sitiens* plants after inoculation with *B. cinerea* (p<0.001) with a fold change of at least 1.5.

Unigene ID	Gene description	ESTs	FC pathogen	FC mock	GO annotation
Genes differentially expressed in wild type after inoculation with *B. cinerea*					
SGN-U143866	P-rich protein EIG-I30, extensin	1	2.33	-1.18	Cell wall related
SGN-U161846	Glycine rich protein, extensin	1	4.64	1.87	Cell wall related
SGN-U147913	Cell wall protein	1	3.27	1.6	Cell wall related
Genes differentially expressed in *sitiens* after inoculation with *B. cinerea*					
SGN-U143332	Proteinase inhibitor type II CEVI57 precursor	1	8.1	1.3	No ontology
SGN-U144237	Hydroxyproline-rich glycoprotein	1	2.8	-1.7	Cell wall related
SGN-U147362	Serine-rich protein	1	4.9	1.2	No ontology
SGN-U143866	P-rich protein EIG-I30, extensin	2	12.7	3.8	Cell wall related
SGN-U144656	Pathogenesis-related protein 1A1	3	32	10.2	Biotic stress
SGN-U143838	Pathogenesis-related protein PR-1	3	17.9	5.8	Biotic stress
SGN-U143303	Lipoxygenase A	1	4.5	1.6	Hormone metabolism
SGN-U145531	Expressed protein	3	3.5	1.2	Hormone metabolism
SGN-U144553	Miraculin homolog	3	4.4	1.8	Biotic stress
SGN-U154970	DnaJ domain-containing protein	1	4.9	2.1	Abiotic stress
SGN-U144826	Pathogenesis-related protein STH-2	1	2	-1.2	Biotic stress
SGN-U143809	Cinnamic acid 4-hydroxylase	1	2.1	-1.1	Secondary metab. , lignin biosynthesis
SGN-U144200	Cytochrome P450 76A2	1	3	1.3	Misc. cytochrome P450
SGN-U143841	Putative peroxidase	1	2.1	1	Misc. peroxidases
SGN-U146275	Putative protein kinase	1	1.7	-1	Posttranslational modification
SGN-U149410	GTP-binding protein Rab11e	1	1.5	-1	Signalling
SGN-U144528	Ethylene-responsive protein-related	1	2	1.1	Hormone metabolism
SGN-U145664	Suberization-associated anionic peroxidase 2	1	1.6	-1.1	Misc. peroxidases
SGN-U143930	Bifunctional lysine-ketoglutarate reductase/saccharopine dehydrogenase	2	3.7	2.2	Amino acid metabolism
SGN-U144114	Expressed protein	1	2.7	1.7	No ontology
SGN-U151083	Unknown	1	1.6	1	Transcription regulation
SGN-U148425	bHLH protein	1	1.8	1.2	Transcription regulation
SGN-U156084	Phospholipid/glycerol acyltransferase	1	1.7	1.2	No ontology
SGN-U144589	Ser/Thr specific protein phosphatase 2A B regulatory subunit β	1	1.6	1.2	Protein degradation
SGN-U146465	Hsr201 protein	1	-2.5	-1.4	Biotic stress
SGN-U145272	Wound-induced protein Sn	1	-3.1	-1.3	Abiotic stress

FC: Fold change of expression 8 hpi compared to 0 hpi. When more than one EST for the same gene is present in the data, the EST with the highest FC is presented. Genes are ranked according to the largest difference in expression at 8 hpi between mock and pathogen inoculation.

DISCUSSION

Here, we show that the very high level of resistance to *B. cinerea* in ABA-deficient *sitiens* tomato plants coincided with a prompt localised accumulation of H_2O_2 that could be prevented by exogenous ABA application. H_2O_2 accumulation in *sitiens* was accompanied by an increase of extracellular peroxidase activity and was located in the epidermal cell layer where it caused both cell wall modification and an HR-like response. Although ROS have a dual role after pathogen attack, acting as key defence compounds against biotrophic pathogens on the one hand, but serving as the molecules by which necrotrophs exploit these responses on the other hand, we demonstrate that a timely hyperinduction of H_2O_2-dependent defences can be effective in arresting the necrotroph *B. cinerea*.

Transcript profiling analysis revealed that defence-related transcript accumulation prior to inoculation is higher in *sitiens* than in wild type. Moreover, further elevation of defence gene expression is stronger after *B. cinerea* attack, both in number of genes and their expression levels. Our results indicate that lower basal ABA levels result in a pre-alerted state of defence in *sitiens*, allowing the mutant to respond earlier and more strongly to pathogen challenge. Recently, Mohr and Cahill (2007) reported that addition of exogenous ABA induced susceptibility in a normally incompatible interaction between Arabidopsis and *Pseudomonas syringae* pv. *tomato* by suppression of lignin and SA accumulation. Moreover, treatment with ABA suppressed the expression of many defence-related genes (Mohr and Cahill, 2007). Anderson et al. (2004) similarly observed higher basal transcript levels of defence-related genes in ABA-deficient Arabidopsis mutants. Moreover, the *abi2-1* mutant was more resistant against the necrotrophic fungus *Fusarium oxysporum*. An obvious genetic or biochemical link between ABA and the higher resistance level is not available. Pti5, a tomato pathogen-inducible ethylene response element-binding protein-like transcription factor, whose expression is higher in *sitiens* than in wild type prior to inoculation, can provide a link between ABA deficiency and pathogen-induced gene activation. Pti5 is expressed specifically during biotic but not abiotic or hormonal stresses, suggesting a specific role for Pti5 in plant defence against pathogens (Thara et al., 1999). Overexpression of Pti5 in tomato enhances resistance against *Pseudomonas syringae* pv. *tomato* and primes for expression of osmotin, β-glucanase, and catalase (He et al., 2001). Interestingly, an osmotin-like protein (SGN-U144488) and β-glucanase (SGN-U143416) (Table II-I) are also highly expressed in *sitiens* prior to inoculation. In Arabidopsis, SA-regulated PR protein genes were regulated by Pti5 (Gu et al., 2002). Hence, high expression

levels of PR proteins in *sitiens* before and after *B. cinerea* inoculation might be a result of Pti5-mediated regulation. Otherwise, the fact that the *sitiens* mutant can be considered to permanently suffer from drought stress because of the lack of ABA-mediated stomatal regulation (Nagel et al., 1994) might explain the higher basal expression of defence-related genes in the mutant, because abiotic stress can cause priming for pathogen defence (Conrath et al., 2002).

Consistent with Audenaert et al. (2002a) we found some genes transcriptionally activated by *B. cinerea* that are involved in SA-dependent signalling (e.g. PR1-protein) or the phenylpropanoid pathway (e.g. cinnamic acid 4-hydroxylase) (Table II-II, Table II-SI). It was shown that *nahG* tomato plants that are unable to accumulate SA are slightly more susceptible to *B. cinerea*. Moreover, PAL activity, implicated in SA biosynthesis, was higher upon inoculation in *sitiens* than in wild-type plants (Audenaert et al., 2002a). Opposing results to these were found in Arabidopsis, where *nahG* mutants showed no susceptibility to *B. cinerea* (Thomma et al., 1998; Veronese et al., 2004) and mutants with induced or constitutively activated systemic acquired resistance (SAR) and SAR gene expression generally were more susceptible to *B. cinerea* (Govrin and Levine, 2002, Kachroo et al., 2001). However, Govrin and Levine (2002) also reported that removal of basal SA accumulation by expression of the *nahG* gene or by infiltration of 2-aminoindan-2-phosphonic acid, increased *B. cinerea* disease symptoms. In addition, SA accumulation was shown to play a role in local resistance to *B. cinerea* inoculation in Arabidopsis mutants that constitutively express SAR but do not have a cell death phenotype (Ferrari et al., 2003). Finally, it was shown before that the role of SA signalling in defence against necrotrophic pathogens might differ according to the host plant (Achuo et al., 2004).

Necrotrophic fungi such as *B. cinerea* feed upon dead plant tissue, implying that the pathogen is able to kill host cells during infection. *Botrytis cinerea* induces ROS formation in plants resulting in hypersensitive cell death that facilitates fungal colonization (Elad, 1992; Govrin and Levine, 2000; Govrin et al., 2006; Schouten et al., 2002; von Tiedemann, 1997). We propose that timing, localisation, and function of the increase in ROS are crucial in its role on *B. cinerea* development. Under our experimental conditions, H_2O_2 in wild-type tomato started to accumulate after 24 h in mesophyll tissue colonized by *B. cinerea* and was associated with spreading cell death. In *sitiens* however, H_2O_2 accumulation was observed from 4 hpi specifically in the epidermis where it caused cell wall modification, which is known to be an essential part of the plant's defence response and to pose physical barriers to invading

pathogens. From 12 hpi, intracellular H_2O_2 was present in HR-like epidermal cells in *sitiens*. Considering that these HR cells in *sitiens* might result from an overaccumulation of defence compounds and that, given the low number of cells involved, total nutrient release will be limited, the HR-like cells can be expected to have only a small effect on total fungal development. It seems unlikely that the H_2O_2 accumulation in *sitiens* has a direct fungitoxic effect on *B. cinerea*, because this pathogen possesses an array of enzymes to protect itself against damage by ROS and has the ability to grow in relatively high concentrations of H_2O_2 (Schouten et al., 2002; Lyon et al., 2004). Moreover, neither in *sitiens* nor in wild type, we observed signs of hyphal death of the invading fungus.

The importance of H_2O_2 accumulation in the resistance to *Botrytis* was shown by an increased susceptibility in *sitiens* after application of different antioxidants. In wild-type plants, catalase treatment had no significant effect on disease development, but in *sitiens* it partially eliminated H_2O_2 accumulation and increased the number of spreading *B. cinerea* lesions, although wild-type control levels were not reached. Catalase has a specific, well-characterized mode of action and eliminates H_2O_2 generated in the cell wall and the plasma membrane (Mellersh et al., 2002). The fact that catalase did not remove all extracellular H_2O_2 nor fully restored susceptibility in *sitiens*, might be explained by inadequate uptake or activity of the enzyme. Profuse *B. cinerea* symptom development was observed on both genotypes after ascorbic acid treatment. This strong antioxidant not only removes the pathogen-induced H_2O_2 accumulation in *sitiens*, but appears to disrupt all defences, including the ones that are normally capable of retarding pathogen development in wild-type tomato. It can not be excluded that ascorbate treatment has provoked unwanted changes and disrupted normal plant metabolism, as it is known that ascorbate serves as an important redox compound controlling redox homeostasis and can have effects on plant growth, photosynthesis, cell cycle and hormone production (Noctor, 2006). In addition to antioxidant treatments, inhibition of ROS generation with DPI increased susceptibility in *sitiens* to wild-type levels. Together, these findings point to the existence of additional H_2O_2-dependent defence mechanisms in the *sitiens* mutant that are not adequately expressed in wild-type tomato. Our observations are consistent with other studies that also have demonstrated that accumulation of ROS can be essential in a successful defence against *B. cinerea*. Previously, resistance of tomato plants against *B. cinerea* infection has been demonstrated to result from early stimulation of H_2O_2 and superoxide radical generation by NADH peroxidase and superoxide dismutase in the apoplastic space (Patykowski and Urbanek, 2003). Moreover, while an aggressive isolate of *B. cinerea* induces an expanding pale brown necrosis, a non-aggressive isolate is arrested by a biphasic oxidative burst and

HR-like necrosis on bean leaf tissue (Unger et al., 2005).

Two major mechanisms of ROS production in plants upon pathogen attack have been described: membrane-bound NADPH oxidases, inhibited by DPI, and cell wall peroxidases, inhibited by azide (Lamb and Dixon, 1994; Torres et al., 2006). While the effect of azide on pathogen induced ROS accumulation could not be established due to its antimicrobial effect (data not shown), application of DPI to *sitiens* leaf discs inhibited H_2O_2 production upon *B. cinerea* inoculation and increased susceptibility, pointing to the involvement of NADPH oxidases as a source of ROS in *sitiens*. However, as the specificity of DPI to exclusively block NADPH oxidases has been questioned (Bolwell et al., 1998), we cannot make conclusive statements regarding the source of *sitiens* ROS generation.

Early H_2O_2 accumulation at the site of plant-fungal contact has been shown to be decisive for the outcome of several tomato-pathogen interactions. H_2O_2 is critical to determine resistance of tomato to *Cladosporium fulvum* (Borden and Higgins, 2002). The same was found for the interaction of tomato with the anthracnose fungus (*Colletotrichum coccodes*) (Mellersh et al., 2002) and with the powdery mildew fungus (*Oidium neolycopersici*) (Mlíčková et al., 2004). In these interactions, penetration failure and resistance result from an early accumulation of H_2O_2 in the anticlinal epidermal cell walls in close contact with the fungal structures. The *sitiens* response to *B. cinerea* shows striking analogy in timing and localization of H_2O_2 accumulation with these tomato defence reactions to biotrophic and hemi-biotrophic pathogens, but are even more forcefully triggered and therefore are effective in arresting development of the necrotroph *B. cinerea*.

Fortification of the cell wall is a multistep process that involves H_2O_2-mediated cross-linking of preformed molecules and induction of transcription-dependent defences (Bradley et al., 1992; Ribeiro et al., 2006). In our system, expression of defence-related cell wall genes was observed early after inoculation and coincided with microscopically visible cell wall modification (8 hpi). Genes encoding hydroxyproline and glycine-rich proteins, structural plant cell wall proteins that can be cross-linked (Showalter, 1993), are expressed more strongly in *sitiens* than in wild type. Also, genes encoding enzymes involved in the phenylpropanoid biosynthesis pathway or in the peroxidative incorporation of these phenolic compounds into the cell wall, such as the suberization-associated peroxidase and the cinnamic acid 4-hydroxylase, are transcriptionally activated. Furthermore, microarray data also revealed a role for enzymes that function in pectin and cellulose modification (e.g. xyloglucan endotransglycosilases and pectinesterases). In view of the infection process of *B. cinerea*, the anticlinal cell wall of the

epidermis is a promising site for reinforcement (Fig. II-9). After breaching the plant's cuticle, *B. cinerea* hyphae grow within the epidermal outer periclinal cell wall before invading the underlying tissue. This penetration to the mesophyll layer occurs intercellularly between epidermal cells and implies the collapse of anticlinal cell walls and dissolvement of the middle lamella (Clark and Lorbeer, 1976). Hence, obstruction at the anticlinal cell walls blocks the passage for *B. cinerea* to the underlying tissue. Besides forming a mechanical barrier to physical fungal penetration, cell wall reinforcements are known to decrease the susceptibility to cell wall-degrading enzymes, to impede nutrient diffusion to the pathogen, and to possibly restrict diffusion of toxins (Brisson et al., 1994; Bestwick et al., 1998; van Kan, 2006). Again, the anticlinal wall is a supreme target to hinder cell wall degradation during the early invasion of the epidermal layer, because anticlinal walls are very rich in pectin, and pectin decomposition and consumption are essential in the development of *B. cinerea* on all hosts (van Kan, 2006). Moreover, Kars et al. (2005a) showed that action of *B. cinerea* pectinases, needed for the growth in the anticlinal walls, is required for normal primary lesion formation. Although a number of studies indicate the importance of cell wall modification in the defence against *Botrytis* on several hosts (Mansfield and Hutson, 1980; Mitchell et al., 1994; McLusky et al., 1999; van Baarlen et al., 2004b), the requirement of localised ROS accumulation in this response to *Botrytis* was, to our knowledge, never presented before.

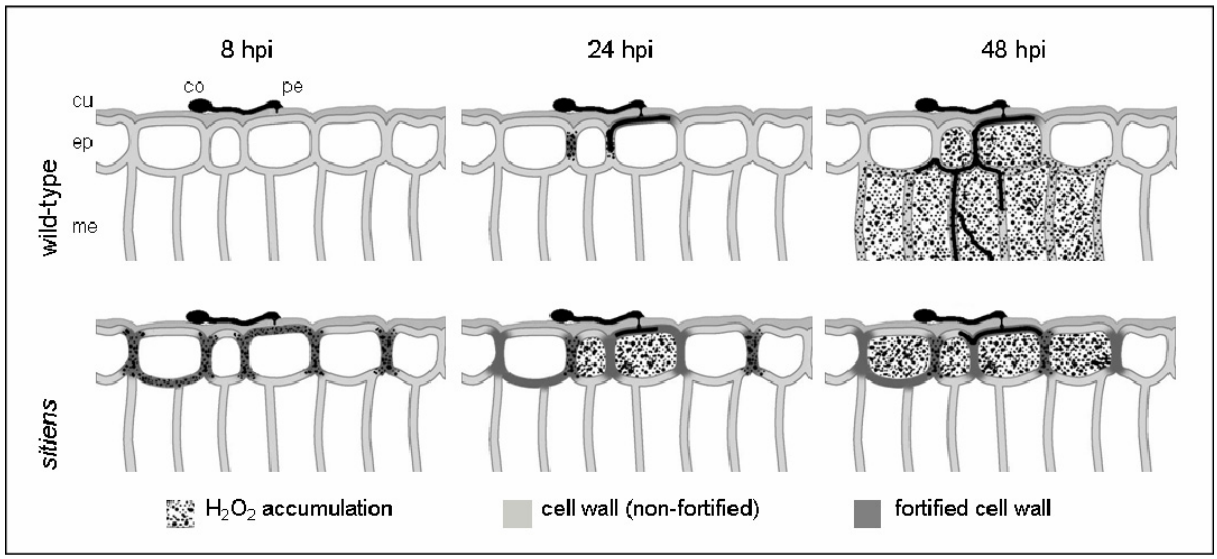

Figure II-9. Schematic model of the location and sequence of fungal growth, H_2O_2 accumulation, and cell wall modification in wild-type and *sitiens* leaf tissue infected with *B. cinerea*.
Wild-type tissue displays only very few zones with H_2O_2 accumulation or cell wall modification until 24 hpi. Between 24 and 48 hpi, strong H_2O_2 accumulation appears mainly in the mesophyll cell layer and is associated with cell death and fungal spreading. In *sitiens*, H_2O_2 accumulation and modifications coincide in a first phase (around 8 hpi) mainly in the anticlinal walls of epidermal cells. At later time points (24 and 48 hpi), fungal growth inside *sitiens* is blocked by these modifications and is thereby limited to the epidermal outer periclinal wall. Intracellular H_2O_2 is mainly restricted to HR-like epidermal cells. Abbreviations: co, conidium; cu, cuticula; ep, epidermal cell layer; me, mesophyll cell layer; pe, point of penetration.

The relative contribution to *B. cinerea* resistance of cell wall modifications and concomitant production of secondary antimicrobial metabolites has often been questioned (reviewed by van Baarlen et al., 2004a). In our interaction, we cannot exclude a role for secondary defence compounds in *sitiens*, because there are several reports dealing with phytoalexin production during *B. cinerea* infection and ROS can serve as a signal for phytoalexin production (Thoma et al., 2003; van Baarlen et al., 2004b). However, due to the rapid and extensive nature of the cell wall modifications in *sitiens*, we propose that wall strengthening will have an important effect in delaying fungal colonization and progress, with antimicrobial secondary metabolites acting during subsequent stages of plant defence.

As opposed to the generally accepted theorem that plant defence-related ROS formation aids necrotrophs in their pathogenicity, we state that a timely production of H_2O_2 and a fast induction of defence responses including cell wall modifications are efficient in protecting the *sitiens* tomato mutant against *B. cinerea*. We propose that resistance due to low ABA content originates from higher basal defence-related transcript accumulation and subsequent fast and strong defence activation upon pathogen challenge.

MATERIALS AND METHODS

Plant material, exogenous ABA application and antioxidant treatments

Tomato (*Solanum lycopersicum* L., previously *Lycopersicon esculentum* Mill.) *sitiens* mutants (Taylor et al., 1988; Taylor et al., 2000), provided by Prof. M. Koornneef (Wageningen University, The Netherlands) and the corresponding wild-type cultivar Moneymaker were grown in potting compost soil (Substrat 4; Klasmann-Deilmann, Groß Hesepe, Germany) at 22°C. The plants were raised in a growth chamber with 75% relative humidity in a 16 h/8 h light-dark regime. ABA treatment was performed as described by Achuo et al. (2006). Plants were sprayed until run-off with 100 µM of ABA twice a week during their development. After 4 to 5 weeks, when seedlings were at the 5th leaf stage, leaf discs were punched out of the tertiary leaves with a 1-cm diameter cork bore and placed floating with the adaxial side up in 24-well plates (VWR, Leuven, Belgium). Each well was filled with 1.5 ml of distilled water or, if indicated, with 1.5 ml of the solution of the chemical treatments. The treatments used consisted of 5 mM of ascorbic acid (Sigma-Aldrich, St. Louis, MO), 1100 U/ml catalase (Sigma-Aldrich), 50 µM diphenilene iodonium (DPI) (Sigma-Aldrich) and distilled water as control. The discs of

the ABA-treated plants were placed in distilled sterile water as in the control treatment. For transcription profiling, whole tertiary leaves of 5-week-old plants were used for inoculation.

Fungal material and inoculation method

Conidia of *B. cinerea* strain R16 (Faretra and Pollastro, 1991) were obtained as described by Audenaert et al. (2002a). The conidial suspension was centrifuged for 10 min at 10 000xg. After removal of the supernatant and re-suspension of the conidia in distilled water, an inoculation suspension was prepared containing $6.25x10^4$ conidia/ml in 16.7 mM KH_2PO_4 and 25 mM glucose. Conidia were pregerminated for 2 h in the inoculation suspension at 22°C. For leaf disc assays, two 5-µl droplets were used to inoculate each tomato leaf disc. The plates containing the leaf discs were incubated at 22°C under dark conditions. Symptoms were evaluated after 4 days. Each inoculation droplet was classified as a spreading or non-spreading lesion and the data were analysed with a binary logistic regression. At least 72 inoculation drops were evaluated for each treatment. For RNA extraction, a spray inoculation was used in order to get a uniform infection covering the total leaf area. Leaves were sprayed with an inoculation solution containing 5.10^4 spores/ml, 0.01 M KH_2PO_4 (pH 5), and 6.67 mM glucose, and placed in plastic trays with high humidity according to Audenaert et al. (2002a) and incubated at 22°C under dark conditions. Mock inoculation consisted of spraying plant leaves with a solution of 0.01 M KH_2PO_4 (pH 5) and 6.67 mM glucose.

Visualisation of defence responses

To compare the defence responses of *sitiens* and wild-type leaf tissue, different staining techniques were used and evaluation was done macroscopically and microscopically. Leaf discs were inoculated with *B. cinerea* as described above and sampled at 4, 8, 12, 16, 20, 24, 48, and 72 hpi by clearing and fixating in 100% ethanol. For each time point, at least three discs originating from different plants of *sitiens* and wild type were used. For H_2O_2 accumulation, staining was according to the protocol of Thordal-Christensen et al. (1997). Three hours before each sampling time point, tomato leaf discs inoculated with *B. cinerea* were placed under light conditions and floated in a solution of 1 mg/ml 3,3'-diaminobenzidine (DAB)-HCl (pH 4). Polymerization of the DAB molecule at the site of H_2O_2 and peroxidase accumulation results in a brown reddish colour that is macroscopically visible and because of the high spatial and

temporal distribution of the oxidized DAB molecule, it can be visualized using bright-field microscopy. A subset of the ethanol-fixed DAB-stained samples were embedded in Technovit 7100 histo-embedding medium (Heraeus Kulzer, Wehrheim, Germany) according to the manufacturer's instructions and semi-thin cross-sections (4 µm) were cut with a Leica RM2265 motorized rotary microtome (Leica Microsystems, Nussloch, Germany). To detect superoxide, leaf discs were floated in 0.05% nitroblue tetrazolium (NBT) for 30 min before fixation in 100% ethanol, according to the protocol of Doke (1983). For protein cross-linking, staining was performed as described by Mellersh et al. (2002). Ethanol-fixed samples were placed in 1% SDS at 80°C for 24 h and stained with 0.1% Coomassie blue in 40% ethanol/10% acetic acid for 15 min and subsequently washed in 40% ethanol/10% acetic acid. To visualize cell wall modifications, safranin-o staining was according to Lucena et al. (2003). Leaf discs were incubated in 0.01% safranin-o in 50% ethanol for 3 min. Accumulation of phenolics was detected by staining with 0.05% toluidine blue in citrate/citric acid buffer (50 mM, pH 3.5) (Mellersh et al. (2002). Fungal structures were stained with 0.02% trypan blue in lactophenol. After staining, leaf discs were mounted in 50% glycerol. Fluorescence and bright-field microscopy were performed with an Olympus BX-51 microscope and images were captured with a ColorView III camera and edited with the software package CELL-F (Olympus Soft Imaging Solutions, Münster, Germany).

Extracellular peroxidase activity was measured with the TMB assay based on Ros Barceló (1998). Leaf discs, inoculated with two drops of a *B. cinerea* conidial suspension or with the appropriate mock solution, were fixed in ethanol. After subsequent washing in distilled water, the discs were incubated in 1.5 ml of a 50 mM tris-acetate buffer (pH 5.0) containing 0.1 mg/ml 3,5,3',5'-tetramethylbenzidine (TMB) and 0.03% H_2O_2 for 20 min. Peroxidase activity of the discs was determined by measuring the absorbance of the incubation solution at 654 nm.

Sampling and RNA preparation

Leaf samples were taken at 0 and 8 hpi. Two leaflets per leaf were excised and immediately frozen in liquid nitrogen. Three days after inoculation, infection levels on the three remaining leaflets were scored. For each genotype/treatment combination (i.e. wild type mock, wild type infected, *sitiens* mock, and *sitiens* infected), four leaflets were sampled and pooled. Total RNA was prepared from the sample pools with RNeasy Plant Mini kit (Qiagen, Hilden,

Germany), according to the manufacturer's instructions. Total RNA quality was checked on an agarose gel, and concentrations were determined with a ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE).

Microarrays

The tomato TOM1 microarray used was obtained from the Center for Gene Expression Profiling of the Boyce Thompson Institute and consisted of 12 899 EST clones representing 8600 independent tomato genes. The functional annotation of the genes related to the spotted cDNAs can be viewed at http://bti.cornell.edu/CGEP/CGEP.html.

Target labelling and hybridizations

Target labelling and hybridizations were carried out at the VIB MicroArray Facility (Leuven, Belgium; http://www.microarrays.be). Total RNA (5 μg) of each sample was reverse transcribed to double-stranded cDNA and further amplified according to a modified version of the protocol described in Puskás et al. (2002) (http://www.microarrays.be/service.htm). Subsequent Cy5 and Cy3 (GE-Healthcare, Little Chalfont, UK) labelling, hybridization, posthybridization washing, and scanning were performed according to protocols accessible through the website (http://www.microarrays.be/service.htm).

Experimental design and statistical analysis of the microarray data

We constructed a loop design (Supplementary Fig. IV-S3) consisting of 14 two-dye TOM1 microarray experiments, in which two independent pools of four leaflets harvested from 5-week-old mock-sprayed and spray-inoculated plants at 0 and 8 hpi were compared. The expression data were analyzed in two steps: (i) a within-slide analysis aimed at removing variation associated with differential dye responses to binding and scanning, as noise; and (ii) a between-slide analysis aimed at estimating the mean differences between treatments (e.g. genotypes and pathogen inoculation treatments) and their standard error. To correct for dye intensity differences, we used the robust scatter plot smoother LOWESS (Yang et al. 2002) as implemented in Genstat (Payne and Lane, 2005), where the response variable is the $\log_2$ ratio of the artefact-removed total foreground Cy3 and Cy5 fluorescence intensities measured at the 12 899 spots. The fraction of the data used for estimating the local LOWESS fit was set at 20%.

For the between-slide analysis, a two-step mixed-model analysis of variance (Wolfinger et al., 2001) was used and variance components were estimated by residual maximum likelihood (REML), as implemented in Genstat (Payne and Lane, 2005) and described previously (Vuylsteke et al., 2005; Nettleton, 2006). Following Wolfinger et al. (2001), the mixed-model analysis on the LOWESS fits to the spot measurements consisted of two steps. First, array and channel effects were removed from the expression responses by a normalization ANOVA model of the form

$$\text{response} = \mu + \text{array} + \text{dye} + (\text{array.dye}) + \text{residual} \qquad (1),$$

where the response variable represents the corrected $\log_2$-transformed Cy3 and Cy5 fluorescence intensity measurements of the 12 899 ESTs. Array models the hybridization effects of each of the 14 microarrays; dye models the effects of the each of the two dyes, and dye by array models the 28 channel effects. Array, dye and dye by array were added as random terms.

In a second step, the residuals from model (1) were analyzed for each of the 12 899 ESTs separately by a mixed model of the following form:

$$\text{residual} = \mu + \text{dye} + \text{replicate} + \text{genotype} + \text{treatment} + (\text{genotype.treatment}) + (\text{genotype.treatment.time}) + \text{array} + \text{error} \qquad (2),$$

partitioning gene-specific variation into gene-specific fixed dye effects, fixed replicate effects, fixed genotype (wild type and *sitiens*) effects, fixed treatment (mock and infected), fixed genotype-specific treatment effects, and fixed genotype-specific treatment effects across time. The random array term models the effects for each spot and equals the (EST.array) interaction effect. These models were fitted by REML and Wald statistics were calculated to assess significance of the fixed effects in the gene model. No further adjustments for multiple testing were done. From the REML analysis, we saved the vector of estimated genotype, treatment and interaction effects with the corresponding estimated variance-covariance matrix, for each gene. Test statistics for contrasts were constructed from the parameter estimates divided by their standard errors. These ratios were supposed to follow approximately a t-distribution, with the degrees of freedom equal to those for the error term in the gene-specific model. On the basis of the t-approximation to the test statistics for the contrasts, *p*-values were calculated.

Quantitative RT-PCR analysis

Poly(dT) cDNA was synthesized from 2 µg of total RNA with Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA) and quantified on a lightCycler480 real-time PCR detection system (Roche Diagnostics, Brussels, Belgium) with the SYBR Green I RT PCR core kit (Roche Diagnostics). PCRs were carried out in triplicate. Gene-specific primer pairs were designed with Beacon Designer 4.0 (Premier Biosoft International, Palo Alto, CA, USA): SGN-U143838: FWD AACCTAGCTGCCGCTTTCCC – REV TTACGCCACACCACCTGAGTATAG; SGN-U143866: FWD CATCAAAGGGTAAGTGCCCAAAGG – REV AAGGCAAACAGCAGCATCAAGATC; SGN-U134316: FWD TCGCCACCAACATTCACATAACAG - REV AGAATGTGATGGCAAGTTGTTCCC; SGN-U144656: FWD GGACGATGGTCTAGCAGCCTATG – REV CAGCACCAGCAGCGTTTAGC; SGN-U144488: FWD TTGTGGTGGAGTCCTGGATTGC – REV TGGCTGTGCATTGAATTGGATGAC; SGN-U147913: FWD CGGCGGTCGTGGTAGAGG – REV AACAACCATTGCGGCAGTACC

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SUPPLEMENTARY MATERIAL

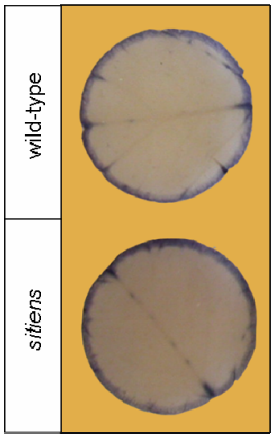

Figure II-S1: Superoxide accumulation detected with nitroblue tetrazolium (NBT) in wild-type and *sitiens* tomato leaf discs inoculated with *B. cinerea*.

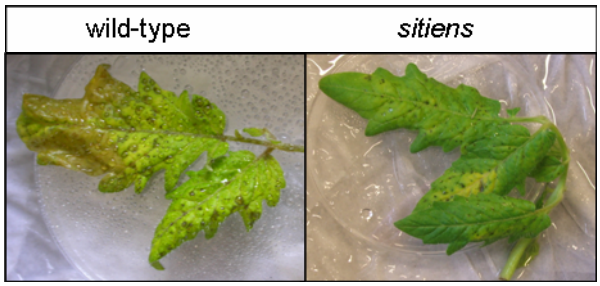

Figure II-S2: Symptom development of wild-type and *sitiens* tomato leaves 3 days after spray inoculation with 5.10^4 *B. cinerea* conidia/mL.

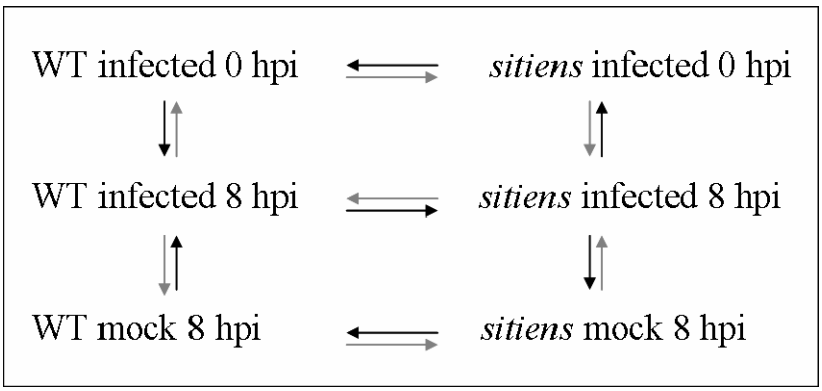

Figure II-S3: Experimental design consisting of 14 TOM1 cDNA arrays to examine transcript levels in RNA samples collected from wild-type and *sitiens* plants, inoculated either with an infection solution or a mock solution, at 0 and 8 hpi.

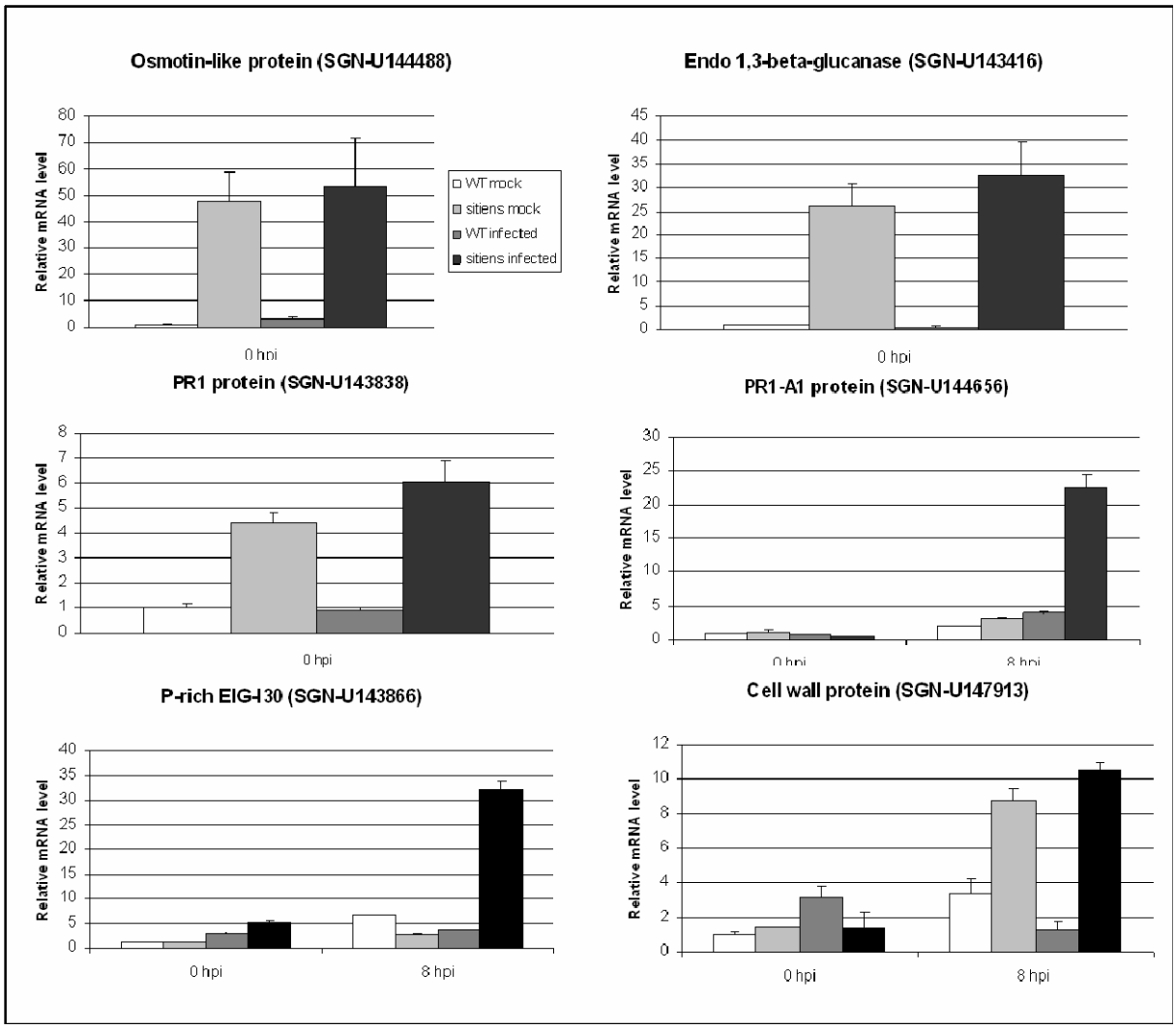

Figure II-S4: Gene expression kinetics of osmotin-like protein (SGN-U144488), endo-1,3-β-glucanase (SGN-U143416), and PR1 protein (SGN-U143838) (Table II-I), PR1A1 protein (SGN-U144656), P-rich protein EIG-I30 (SGN-U143866) (Table II-II) and cell wall protein (SGN-U147913) (Table II-SII) in wild-type and *sitiens* leaves spray-inoculated with 5.10^4 *B. cinerea* conidia/ml.
Gene intensity values were monitored using qRT-PCR in *B. cinerea* and mock-inoculated leaves at 0 and 8 hpi.

Table II-SI: Genes significantly differentially expressed in wild-type and *sitiens* plants after inoculation with *B. cinerea* ($p < 0.01$) with a fold change (FC) of at least 1.5.

Unigene code	Gene description	GO annotation	FC[a]
Wild type			
SGN-U161846	Extensin class I	Cell wall related	4.6
SGN-U146403	Extensin class I	Cell wall related	3.9
SGN-U146173	WD-repeat protein T4I9.10 [*Arabidopsis thaliana*]	Development, unspecified	3.5
SGN-U147913	Cell wall protein [*Nicotiana tabacum*]	Cell wall related	3.3
SGN-U143866	P-rich protein EIG-I30 [*N. tabacum*]	Cell wall related	2.3
SGN-U146578	Non-symbiotic hemoglobin class 1 [*Lycopersicon esculentum*]	Redox heme	1.8
SGN-U145445	Unknown protein [*A. thaliana*]	Unknown	1.8
SGN-U154221	No hits found	Unknown	1.8
SGN-U145571	N-hydroxycinnamoyl/benzoyltransferase [*Ipomoea batatas*]	Secondary metabolism lignin biosynthesis	1.5
SGN-U146894	Expressed protein [*A. thaliana*]	Unknown	1.5
SGN-U143497	Glycolate oxidase -related [*A. thaliana*]	Photorespiration	-1.6
SGN-U154022	Adaptin family [*A. thaliana*]	Cell. organisation	-1.7
sitiens			
SGN-U144656	Pathogenesis-related protein 1A1 precursor (PR-1A1)	Biotic stress	32
SGN-U144656	Pathogenesis-related protein 1A1 precursor (PR-1A1)	Biotic stress	24.9
SGN-U143838	Pathogenesis-related protein PR-1 precursor [*Capsicum annuum*]	Biotic stress	17.9
SGN-U143838	Pathogenesis-related protein PR-1 precursor [*C. annuum*]	Biotic stress	17.7
SGN-U143838	Pathogenesis-related protein PR-1 precursor [*C. annuum*]	Biotic stress	17
SGN-U155861	Expressed protein [*A. thaliana*]	Unknown	14.6
SGN-U143838	Pathogenesis-related protein PR-1 precursor [*C. annuum*]	Biotic stress	13.9
SGN-U143866	P-rich protein EIG-I30 [*N. tabacum*]	Cell wall related	12.7
SGN-U144656	Pathogenesis-related protein 1A1 precursor	Biotic stress	10.4
SGN-U160528	Thaumatin homolog NP24 precursor	Abiotic stress	9.7
SGN-U143414	NP24 protein precursor (Pathogenesis-related protein PR P23)	Abiotic stress	9.6
SGN-U143332	Proteinase inhibitor type II CEVI57 precursor	No ontology	8.1
SGN-U143414	NP24 protein precursor (Pathogenesis-related protein PR P23)	Abiotic stress	6.4
SGN-U143414	NP24 protein precursor (Pathogenesis-related protein PR P23)	Abiotic stress	5.7
SGN-U144111	Pepper esterase [*C. annuum*]	No ontology	5.5
SGN-U147362	Serine-rich protein	No ontology	4.9
SGN-U154970	DnaJ domain-containing protein [*A. thaliana*]	Abiotic stress	4.9
SGN-U143337	Endochitinase 3 precursor	Signaling in sugar and nutrient physiology	4.7
SGN-U143303	Lipoxygenase A	Hormone metabolism	4.5
SGN-U144553	Miraculin homolog, root-knot nematode-induced - tomato	Biotic stress	4.4
SGN-U145219	Arginase [*A. thaliana*]	Amino acid metabolism	4.2
SGN-U152643	Avr9/Cf-9 rapidly elicited protein 194 [*N. tabacum*]	Biotic stress	3.9
SGN-U143930	Bifunctional lysine-ketoglutarate reductase/saccharopine dehydrogenase [*Gossypium hirsutum*]	Amino acid metabolism	3.7
SGN-U143930	Bifunctional lysine-ketoglutarate reductase/saccharopine dehydrogenase [*G. hirsutum*]	Amino acid metabolism	3.6
SGN-U145531	Expressed protein [*A. thaliana*]	No ontology	3.5
SGN-U148573	C2 domain-containing protein [*A. thaliana*]	Signaling calcium	3.2
SGN-U143414	NP24 protein precursor (pathogenesis-related protein PR P23)	Abiotic stress	3.1
SGN-U143868	P-rich protein EIG-I30 [*N. tabacum*]	Cell wall related	3.1
SGN-U144328	Aspartate transaminase AAT1 peroxisomal/glyoxysomal	Amino acid metabolism	3
SGN-U144200	Cytochrome P450 76A2	Misc. cytochrome P4503	3
SGN-U147278	Putative β-alanine-pyruvate aminotransferase	Amino acid metabolism	2.9
SGN-U144553	Miraculin homolog, root-knot nematode-induced [*L. esculentum*]	Biotic stress	2.8
SGN-U145531	Expressed protein [*A. thaliana*]	No ontology	2.8
SGN-U144237	Hydroxyproline-rich protein	Unknown	2.8
SGN-U144553	Miraculin homolog, root-knot nematode-induced [*L. esculentum*]	Biotic stress	2.7
SGN-U144114	Expressed protein [*A. thaliana*]	Hormone metabolism	2.7
SGN-U155632	Ovule development protein, putative [*A. thaliana*]	Transcription regulation	2.6
SGN-U144480	Translocase of chloroplast 34	Signaling G-proteins	2.3
SGN-U143609	Organic anion transporter-like protein [*A. thaliana*]	Transport unspecified cations	2.3
SGN-U143841	Putative peroxidase [*Solanum tuberosum*]	Misc. peroxidases	2.1
SGN-U158148	BAC19.4 [*L. esculentum*]	Unknown	2.1
SGN-U143809	Cinnamic acid 4-hydroxylase [*C. annuum*]	Secondary metabolism lignin biosynthesis	2.1
SGN-U148351	MADS-box transcription factor CDM51	Transcription regulation	2.1
SGN-U144528	Ethylene-responsive protein-related [*A. thaliana*]	Hormone metabolism	2
SGN-U144826	Pathogenesis-related protein STH-2	Biotic stress	2
SGN-U143981	Peptidyl-prolyl isomerase protein [*A. thaliana*]	Cell cycle	2
SGN-U145531	Expressed protein [*A. thaliana*]	No ontology	1.8
SGN-U150706	Protease inhibitor/seed storage/lipid transfer protein (LTP) family [*A. thaliana*]	Misc. protease inhibitor/seed storage/ lipid transfer protein (LTP) family protein	1.8
SGN-U148425	Hypothetical protein AT4g36930	Transcription regulation	1.8
SGN-U146275	Putative protein kinase [*A. thaliana*]	Posttranslational modification	1.7
SGN-U151197	Peroxidase, putative [*A. thaliana*]	Misc. peroxidases	1.7
SGN-U144145	FRO2 homolog [*A. thaliana*]	Metal handling acquisition	1.7
SGN-U151516	Expressed protein [*A. thaliana*]	No ontology	1.7

SGN-U146067	NADH-ubiquinone oxireductase-related	Mitochondrial electron transport	1.7
SGN-U156084	Phospholipid/glycerol acyltransferase family	No ontology	1.7
SGN-U145664	Suberization-associated anionic peroxidase 2 precursor	Misc. peroxidases	1.6
SGN-U143770	OSJNBa0079M09.4 [*Oryza sativa* (japonica cultivar group)]	Misc. myrosinases-lectin-jacalin	1.6
SGN-U155141	P0436D06.15 [*O. sativa* (japonica cultivargroup)]	Unknown	1.6
SGN-U146189	Expressed protein	No ontology	1.6
SGN-U144218	Extensin	Cell wall related	1.6
SGN-U144589	Ser/Thr-specific protein phosphatase 2A B regulatory subunit β-isoform	Protein degradation	1.6
SGN-U151083	Unknown	Transcription regulation	1.6
SGN-U149410	GTP-binding protein Rab11e	Signaling G-proteins	1.5
SGN-U143371	40S ribosomal protein S4	Protein synthesis	1.5
SGN-U145314	Calnexin 1 (CNX1)	Signaling calcium	1.5
SGN-U147707	60S ribosomal protein L23A	Protein synthesis	1.5
SGN-U144034	Purine permease family	Transport nucleotides	-1.6
SGN-U144112	Thioredoxin H-type 1	Redox thioredoxin	-1.6
SGN-U145579	Similar to *hsr*203J	No ontology	-1.7
SGN-U147097	No hits found	Unknown	-1.7
SGN-U146954	Probable calmodulin-binding protein	Signaling	-1.8
SGN-U147298	Expressed protein	Unknown	-1.8
SGN-U149589	Polyphosphoinositide binding protein Ssh2	DNA unspecified	-1.9
SGN-U143900	Serine/threonine kinase	Posttranslational modification	-1.9
SGN-U148374	Probable bHLH transcription factor	Transcription regulation	-1.9
SGN-U146818	Expressed protein	Unknown	-1.9
SGN-U145579	Similar to hsr203J	No ontology	-1.9
SGN-U143775	Ent-kaurenoic acid oxidase	Hormone metabolism	-1.9
SGN-U146135	Hydrolase, α/β-fold family	No ontology	-2
SGN-U148125	No hits found	Unknown	-2
SGN-U145272	Wound-induced protein Sn-1	Abiotic stress	-2.1
SGN-U144820	Expressed protein [*A. thaliana*]	Unknown	-2.2
SGN-U146334	Cyclic nucleotide-gated calmodulin-binding ion channel	Transport	-2.3
SGN-U154575	Expressed protein	Unknown	-2.4
SGN-U145272	Wound-induced protein Sn-1	Abiotic stress	-2.4
SGN-U146465	Hsr201 protein, hypersensitivity-related	Biotic stress	-2.5
SGN-U145272	Wound-induced protein Sn-1	Abiotic stress	-3.1
SGN-U147270	Isoflavone reductase-related	Secondary metabolism	-3.1
SGN-U143664	Cysteine proteinase	Protein degradation	-3.2
SGN-U143776	24K germin-like protein	Abiotic stress	-26.4

[a] FC of expression 8 hpi compared to 0 hpi.

Table II-SII: Non-redundant list of cell wall-related genes significantly differentially expressed upon *B. cinerea* inoculation at 8 hpi (p<0.05) with a FC of at least 1.5 in *sitiens* plants. FC: Fold change of expression after pathogen challenge compared to mock inoculation.

Unigene ID	Gene description	FC *sitiens*	FC wild type
SGN-U143866	P-rich protein EIG-I30, extensin [a]	12.7	2.3
SGN-U145717	Xyloglucan endotransglycosylase	4.3	3.6
SGN-U143868	P-rich protein EIG-I30, extensin [a]	3.1	1.7
SGN-U143928	Xyloglucan endotransglycosylase (XTR4)	2.9	1.6
SGN-U146403	Extensin class I	2.8	3.9
SGN-U144237	Hydroxyproline-rich protein [a]	2.8	1.3
SGN-U147913	Cell wall protein	2.1	3.3
SGN-U143809	Cinnamic acid 4-hydroxylase	2.1	1.2
SGN-U145571	N-hydroxycinnamoyl/benzoyltransferase	1.7	1.5
SGN-U145664	Suberization-associated anionic peroxidase 2 precursor (TMP2) [a]	1.6	-1.2
SGN-U144218	Extensin [a]	1.6	-1.1
SGN-U147063	Pectinacetylesterase	1.6	1.5
SGN-U147999	Xyloglucan endo-1,4-β-D-glucanase	1.5	1
SGN-U154458	Pectinesterase family	1.5	1.2
SGN-U150615	Xyloglucan endotransglycosylase XET2	-1.6	-2

[a] Genes selected based on literature information.

Chapter

3

Hyperinduction of ROS-Fuelled Cell Wall Fortification in *Sitiens* Blocks *Botrytis cinerea* Progression

Bob Asselbergh, Katrien Curvers and Monica Höfte

Addendum to chapter 2 (Resistance to *Botrytis cinerea* in *sitiens*, an Abscisic Acid-Deficient Tomato Mutant, Involves a Timely Production of Hydrogen Peroxide and Cell Wall Modifications in the Epidermis; *Plant Physiology* 2007, 144: 1863-1877)

Botrytis cinerea is the causal agent of gray mould disease on a broad range of plant species and as a necrotroph, it kills and macerates the host tissue for its own growth. Unlike biotrophic interactions where (specific) resistance is commonly based on a gene-for-gene recognition mechanism, no such resistance mechanism has been found in plant interactions with necrotrophs. Moreover, the hypersensitive response (including membrane depolarization, Ca^{2+}-influx, an oxidative burst and eventually cell death), which is induced upon pathogen recognition, is known to be effective against biotrophic pathogens, but was shown to be in favour of necrotrophic development because of the oxidative burst and resulting cell death (Govrin and Levine, 2000). In our study (Asselbergh et al., 2007), we suggested that timing and localization of the oxidative burst are of great importance for its decisive role in the interaction between tomato and *B. cinerea*. Our investigations indicated that early triggering of the oxidative burst can induce resistance against a necrotrophic pathogen, in case *Botrytis cinerea*.

The oxidative burst and hypersensitive response (HR) are essential for plant resistance to a wide range of biotrophic pathogens, but their role in the defence against necrotrophs is less obvious (Glazebrook, 2005). Colonisation of plants by *Botrytis cinerea* is assisted by programmed cell death (PCD) triggered by reactive oxygen species (ROS) formation, which can be produced by fungal enzymes or induced in the plant by pathogenicity factors. In addition, *B. cinerea* possesses a sophisticated enzymatic machinery to protect itself from damage by ROS (Govrin and Levine, 2000; Lyon et al., 2004). Together, these findings have led to the interpretation that an oxidative burst is not effective against or even exploited by *B. cinerea*.

Our present study (Asselbergh et al., 2007) adds some nuances to this view: the hyperinduction of hydrogen peroxide (H_2O_2)-dependent defence in the ABA-deficient *sitiens* tomato mutant led to a high level of resistance in which *B. cinerea* is blocked in the early stages of its development. A pharmacological approach was used to disturb the early H_2O_2 production in *sitiens*, which resulted in restoration of the susceptibility. Moreover, microscopic evidence confirmed that rapid H_2O_2-fuelled anticlinal wall reinforcements and HR-like cellular reactions in the epidermal cell layer hinder fungal progression to the underlying tissue (Fig. III-1). Immunological detection of pectic cell wall components using JIM5 antibody (Knox et al., 1990), enabled us to assess pathogen progression in detail by locating pectinolytic cell wall degradation. In wild type, *B. cinerea* was able to degrade the pectin matrix in all leaf cell layers, whereas in *sitiens*, cell wall degradation was typically limited to the outer periclinal walls of the epidermis (Fig. III-1C). This explicitly shows the importance of epidermal anticlinal wall fortifications in obstructing *B. cinerea*, as was previously suggested (Asselbergh et al., 2007).

The apparent dual role of ROS formation in the outcome of interactions with necrotrophs might be determined by several factors. Firstly, ROS formation has multiple functions in defence, but the relative contribution of each function can vary (Lamb and Dixon, 1997). While ROS-induced PCD favours *B. cinerea* and direct antimicrobial activity of ROS will presumably have little effect, the function of ROS formation in cell wall fortification and in defence signalling should prevail in encountering necrotrophs. Secondly, timing, location and extent of ROS accumulation seem to control its effectiveness. H_2O_2 was rapidly produced in epidermal anticlinal cell walls in *sitiens*, but occurred later in susceptible wild-type tomato where it was mainly located in mesophyll tissue (Fig. III-1B).

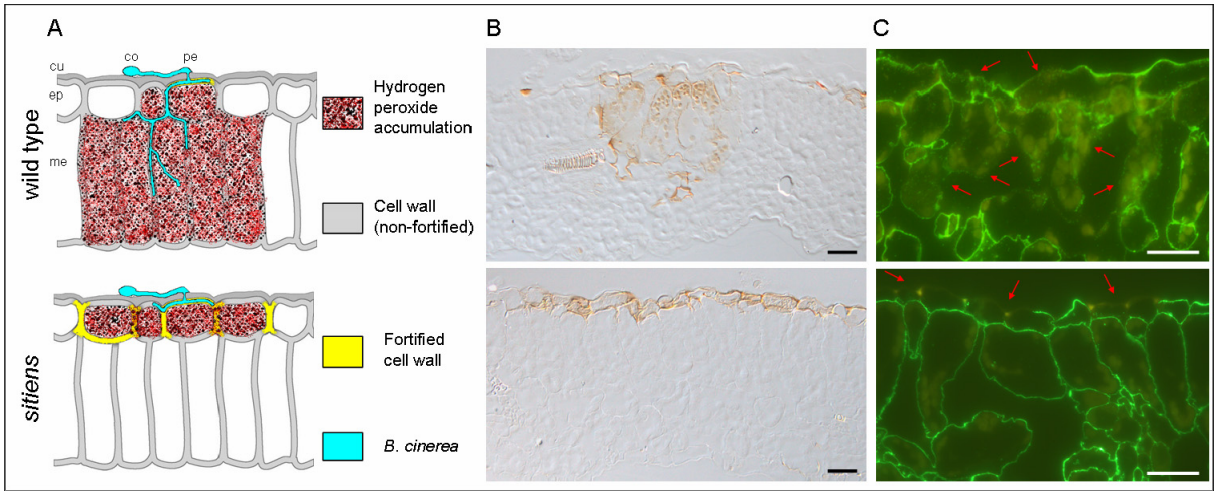

Figure III-1: Defence responses and *B. cinerea* development in *sitiens* and wild-type tomato.
In ABA-deficient *sitiens* tomato, a strong and early accumulation of hydrogen peroxide in the epidermis results in fortification of the anticlinal walls, which blocks fungal cell wall degradation and progression to the underlying mesophyll tissue. In wild-type tomato, hydrogen peroxide accumulation occurs later and is mainly associated with cell death, allowing fungal spreading to the mesophyll.
A: Schematic model of fungal progression, hydrogen peroxide accumulation and cell wall fortification between 32 and 48 hpi. co, conidium; cu, cuticula; ep, epidermal cell layer; me, mesophyll cell layer; pe, point of penetration. B: Hydrogen peroxide accumulation, detected with DAB staining (brown-orange), at 48 hpi. C: Degradation of pectin, detected with monoclonal antibody JIM5 and secondary labelling with FITC, at 32 hpi. Some sites with cell wall degradation are indicated with arrows. The material and methods used are described in chapter 2 (for B) and chapter 6 (for C). Scale bar = 50 µm.

The influence of ABA on plant-pathogen interactions is complex and far from elucidated, and its mode of action seems to differ from that of salicylic acid (SA), ethylene (ET) or jasmonic acid (JA). In the case of ABA, concentration fluctuations upon pathogen attack are relatively small compared to those of JA, SA and ET (Mauch-Mani and Mauch, 2005). Furthermore, JA, SA and ET induce particular signalling pathways leading to activation of specific sets of defence genes (Glazebrook, 2005), whereas ABA has a wide range of effects in different plant-pathosystems (Mauch-Mani and Mauch, 2005; see chapter 1). Recent studies confirm a role for ABA as a global multi-component regulator of responses to pathogen stress. Extensive Arabidopsis gene expression profiling and mutant analysis implicated ABA in the complex regulatory network controlling the response to *B. cinerea* (AbuQamar et al., 2007). Other evidence for a central and decisive role of ABA in response to pathogens was recently provided in the Arabidopsis-*Pseudomonas syringae* interaction (de Torres-Zabala et al., 2007). Effector proteins of *P. syringae* were shown to increase ABA-biosynthesis and signalling which resulted in suppression of the defence response, and this effector-mediated manipulation of the plant hormone homeostasis was suggested to be a major virulence strategy. Moreover, *B. cinerea* has the capacity to produce ABA (Siewers et al., 2006), and its function as a virulence factor was suggested (Kettner and Dörffling, 1995).

When grown under conditions of high relative humidity, ABA deficiency does not

result in major morphological abnormalities. Nevertheless, ABA-deficient plants are unable to cope with drought or cold stress due to the lack of ABA-mediated stomatal regulation (Nagel et al., 1994). The ability of ABA-deficient tomato to block the necrotrophic pathogen *B. cinerea*, as if it were a biotroph, reveals its enormous defensive capacity towards biotic stress. This was also reflected at the transcriptome level, as *sitiens* exhibits higher expression of defence-related genes prior to inoculation and shows a further elevation quickly after *B. cinerea* inoculation, which is shown in Figure III-2. The absence of the suppressive effect of ABA on defence signalling at the onset of pathogen penetration and detection could lead to the higher level of resistance, as was similarly observed by de Torres-Zabala et al. (2007). In Asselbergh et al. (2007), we alternatively hypothesize that the constant sensing of a (low) level of drought stress primes *sitiens* for pathogen stress response. More generally, direct or indirect effects of ABA-deficiency on plant physiology or morphology could influence disease signalling. For example, we are currently investigating the impact of differences in cell wall composition on the augmented defence response in *sitiens*. At last, it can not be excluded that differences in water relations in the ABA-deficient mutant could directly affect pathogen development. Anyhow, it seems that deficiency in ABA results in a global shift towards strong pathogen defence responses at the expense of reduced tolerance to abiotic stress.

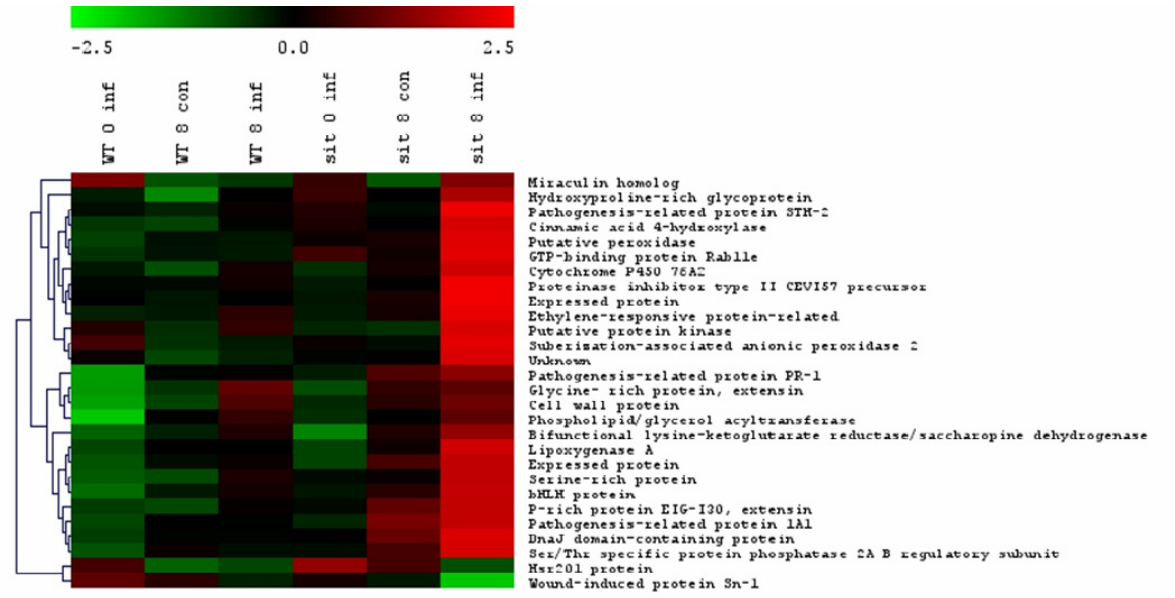

Figure III-2: Hierarchical average linkage clustering tree of 28 genes significantly differentially expressed in wild-type and *sitiens* plants upon *B. cinerea* inoculation ($p<0.001$) with a fold change of at least 1.5 at 8 hours post inoculation (hpi) compared to 0 hpi (corresponding to Table II-II, chapter 2; Asselbergh et al., 2007). Expression patterns in wild-type (WT) and *sitiens* (sit) mock-treated (con) and *Botrytis*-inoculated (inf) plants at 0 and 8 hpi are presented. Red and green correspond to up- and down-regulation, respectively. Expression values for each gene were normalized and median-centred before Hierarchical Linkage Clustering using TMEV v4.0 (Saeed et al., 2003). For more details on data-processing and selection of the displayed genes, see Asselbergh et al., 2007 or chapter 2.

Chapter

4

Basal Tomato Defences to *Botrytis cinerea* Include Abscisic Acid-Dependent Callose Formation

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In Arabidopsis, abscisic acid (ABA) application can induce resistance by priming for callose deposition; this resistance is impaired in ABA-deficient and -insensitive mutants. In tomato, ABA-deficiency causes resistance to *Botrytis cinerea*. Here we show that callose deposition after *B. cinerea* inoculation is weaker in the ABA-deficient *sitiens* tomato mutant compared to the wild type. Inhibition of callose synthesis did not affect resistance in *sitiens*, but caused additional susceptibility in wild type. These findings indicate that callose deposition is not part of *sitiens* defence responses that are effective in blocking *B. cinerea* and suggest that callose deposition only contributes to wild-type basal resistance. Furthermore, also in tomato callose formation is at least in part ABA-dependent. However, it seems that in contrast to Arabidopsis, basal ABA levels in tomato are sufficiently high to prime for callose deposition.

INTRODUCTION

Over the years, reports have accumulated that implicate ABA as a major player in the response to pathogens. The majority of studies propose a role for elevated or basal ABA levels in disease susceptibility, whereas blocking ABA biosynthesis or ABA signalling generally increases resistance (Mohr and Cahill, 2003; Anderson et al., 2004; Koga et al., 2004; Thaler and Bostock, 2004; see chapter 1 or Mauch-Mani and Mauch, 2005 for review). Additional data proving that ABA suppresses defence responses were recently provided by de Torres-Zabala et al. (2007), who showed that successful colonisation of Arabidopsis by *Pseudomonas syringae* is established through manipulation of ABA-responsive gene expression by bacterial effector proteins.

Previously, we have reported on the high levels of resistance to *Botrytis cinerea* in ABA-deficient mutants of tomato (*Solanum lycopersicum*) (Audenaert et al., 2002a). Decrease of ABA content in the *sitiens* mutant resulted in a drastic reduction of the number of spreading lesions. Similarly, ABA-deficiency in tomato increases resistance to the necrotroph *Sclerotinia sclerotiorum* (Rotthier, 2004), the biotrophic fungus *Oidium neolycopersici* (Achuo et al., 2006) and the bacterial pathogens *Pseudomonas syringae* pv. *tomato* (Thaler and Bostock, 2004) and *Erwinia chrysanthemi* (Asselbergh et al., 2008; see chapter 5). We have recently shown that resistance to *B. cinerea* in *sitiens* is caused by a rapid and extensive extracellular accumulation of hydrogen peroxide at the site of infection where it leads to cell wall modification by protein cross-linking and incorporation of phenolic compounds (Asselbergh et al., 2007; see chapter 2).

When plants are attacked by pathogens, a series of protective measures is activated, consisting of both chemical and physical defences. A well studied physical defence is the synthesis of callose, a β-1,3-glucan (Aist, 1976). The synthesis of callose is believed to differ from cellulose synthesis and to be mediated by callose synthases (Verma and Hong, 2001). During fungal infections, callose is deposited in cell wall appositions that form beneath the site of penetration, forming so-called papillae which block pathogen entry. Papillae are apposed on the inner side of epidermal cell walls (between the plasma membrane and the cell wall) and can be formed before penetration of the cell wall (Aist, 1976). The importance of callose formation on the outcome of several plant-pathogen interactions was shown. For example, in barley (*Hordeum vulgare*) papillae formation is essential to prevent penetration of the powdery mildew fungus *Blumeria graminis* (Buschges et al., 1997) and in lettuce (*Lactuca sativa*), depositions of callose around the haustoria of the root pathogen *Plasmopara lactucae-radicis* are essential for

conferring resistance (Stanghellini et al., 1993).

Recent studies have proposed a role for the plant hormone abscisic acid (ABA) in the regulation of callose synthesis after pathogen attack (see Flors et al., 2005 for review). In contrast to the increasing number of studies that implicate ABA as an inducer of plant susceptibility, Ton and Mauch-Mani (2004) found that ABA signalling was required for induced resistance through enhanced callose formation. Treatment of Arabidopsis (*Arabidopsis thaliana*) with β-amino butyric acid (BABA) caused both resistance to the necrotrophic pathogens *Alternaria brassicicola* and *Plectosphaerella cucumerina*, and an earlier and more pronounced callose formation upon inoculation. Both effects were lost in ABA-deficient or ABA-insensitive mutants (*aba1-5* and *abi4-1* respectively) and in the callose deficient mutant *pmr4-1*, which led to the conclusion that BABA-induced resistance against necrotrophic pathogens is based on primed callose accumulation, which is controlled by a new ABA-dependent defence pathway (Ton and Mauch-Mani, 2004). This view was further strengthened after a screen for BABA-response mutants by the same group of authors, which revealed that the BABA-response mutant *ibs3* was affected in the regulation of the zeaxanthin epoxidase gene *ABA1* of the ABA biosynthetic pathway and in BABA-induced resistance and callose deposition (Ton et al., 2005). In addition, a recent study of Kaliff et al. (2007) shows that Arabidopsis resistance to *Leptosphaeria maculans* through the $RLM1_{col}$ pathway is mediated by components of ABA signalling. Moreover, it was shown that ABA influences both callose-dependent and callose-independent resistance (Kaliff et al., 2007).

The objective of the present study was to evaluate the significance of callose deposition in the tomato - *B. cinerea* interaction and to determine whether this defence response is also influenced by ABA in tomato. Callose deposition was compared between ABA-deficient *sitiens* and wild type, and the contribution of callose to the resistant and to the susceptible interaction was assessed after inhibition of callose synthesis with 2-deoxy-D-glucose (2-DDG). Our results suggest that callose formation in tomato depends partly on functional ABA levels and contributes to basal resistance to *B. cinerea*, but not to the high level of resistance that is observed in *sitiens*.

RESULTS

Location of callose deposits in tomato infected with *B. cinerea*

To determine the effect of callose formation on the interaction of tomato with *B. cinerea*, a standardised pathogen inoculation protocol was used in which leaf tissue of five-week-old tomato cv. Moneymaker was inoculated with drops of a *B. cinerea* conidial suspension. Callose was stained with aniline blue and observed under UV excitation. Fixation and decolouration in lactophenol was needed to allow acceptable visualisation of callose (see Materials and Methods). In uninfected or mock-inoculated leaf tissue, no callose was detected except for some small spots in the vascular tissue (Fig. IV-1). In *B. cinerea* inoculated leaf tissue, bright callose deposits were present from about 20 h post inoculation (hpi) beneath the inoculation droplet. In general, deposits could be perceived at three different locations. In most cases, callose was present as single spots corresponding with unsuccessful fungal penetration attempts and was located in the epidermal cells at the site of contact with the fungal germ tube or appressorium-like structure (Fig. IV-2). Besides forming single-spot deposits, some epidermal cells accumulated callose in their anticlinal cell walls. Finally, callose could also be detected in the walls of mesophyll cells at sites where the fungus was successful in penetrating the epidermal cell wall (Fig. IV-2).

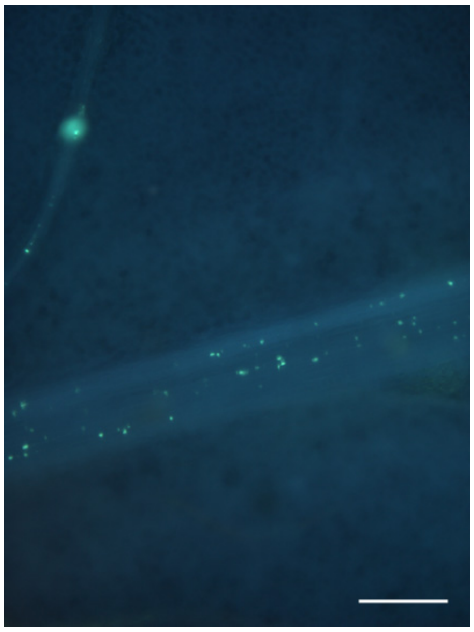

Figure IV-1: Basal callose presence in healthy tomato leaf tissue. Callose was stained with aniline blue and visualised under UV excitation. Bright green callose deposits are present only in the vascular tissue of the leaf. Scale bar = 20 µm.

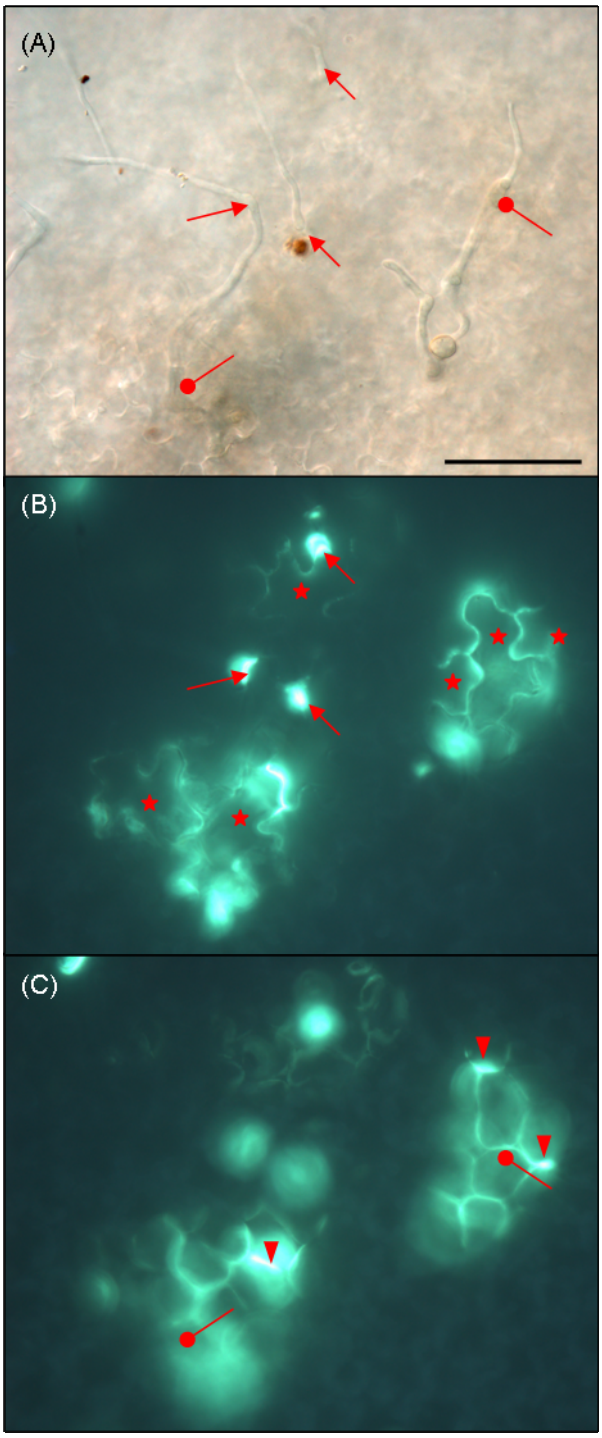

Figure IV-2: Callose formation in tomato leaf tissue inoculated with *B. cinerea*.
Micrographs (A), (B) and (C) are taken with a fixed xy-frame, showing the same position on the leaf. (A): Bright field micrograph showing fungal penetration attempts. (B), (C): Micrographs under UV excitation showing aniline blue-stained callose deposits. In (B), the focus is on the epidermal cell layer, while in (C), the mesophyll cells are in the focal plane.
In (A) and (C), successful fungal penetrations are marked with "blunt arrows", while "sharp arrows" in (A) and (B) mark unsuccessful penetration attempts. Three types of callose deposits could be distinguished: single spots at unsuccessful penetration sites ("sharp arrows" in (B)); callose deposition in epidermal anticlinal cell walls (cells marked with "star" in (B)) and callose deposits in walls of mesophyll cells ("arrow heads" in (C)). Scale bar = 50 µm.

Comparison of callose accumulation between wild-type and ABA-deficient *sitiens* tomato

The ABA-deficient *sitiens* mutant of tomato was previously shown to be very resistant to *B. cinerea* in comparison to the wild-type cv. Moneymaker, with only a low number of *sitiens* inoculation sites that resulted in macerated spreading lesions (Audenaert et al., 2002a). Furthermore, the resistance was shown to be based on a strong and fast activation of defence

responses, including defence gene activation, hydrogen peroxide production and cell wall fortification, blocking *B. cinerea* in the early stages of its development (Asselbergh et al., 2007; see chapter 2). Considering the role of ABA in callose deposition upon pathogen attack, we evaluated the accumulation of callose after inoculation of *sitiens* and wild-type tomato with *B. cinerea*.

No differences were detected between the two tomato genotypes in the onset of callose formation. Both *sitiens* and wild type formed detectable callose deposits around 20 hpi and the accumulation was located at the same sites in *sitiens* as in wild type. However, *sitiens* accumulated a lower amount of callose than wild-type: a smaller proportion of the *sitiens* leaf area contained detectable callose and *sitiens* callose deposits generally stained less intensely compared to the bright and abundant staining in wild type (Fig. IV-3).

Effect of 2-deoxy-D-glucose on *B. cinerea* disease development and callose formation in wild-type and *sitiens* tomato

To evaluate the contribution of callose deposition to tomato defence against *B. cinerea*, we made use of 2-DDG, a known inhibitor of callose production in plants (Jaffe and Leopold, 1984) that was shown to reduce resistance caused by callose formation upon fungal penetration in different plant-pathosystems (Stanghellini et al., 1993; Ton and Mauch-Mani, 2004; Bayles et al., 1990). Application of 25 mM 2-DDG strongly reduced the amount of callose deposited in both wild type and *sitiens* after inoculation with *B. cinerea* (Fig. IV-4). Interestingly, removal of callose deposition by 2-DDG treatment had no significant effect on the resistant response of *sitiens* to *B. cinerea* (Fig. IV-5). On the other hand, blocking callose formation in wild type caused an additional increase in the number of spreading lesions. These results indicate that callose formation does not contribute to the high level of resistance observed in the ABA-deficient *sitiens* mutant, but suggest that callose plays a role in the basal defence of wild-type tomato to *B. cinerea*.

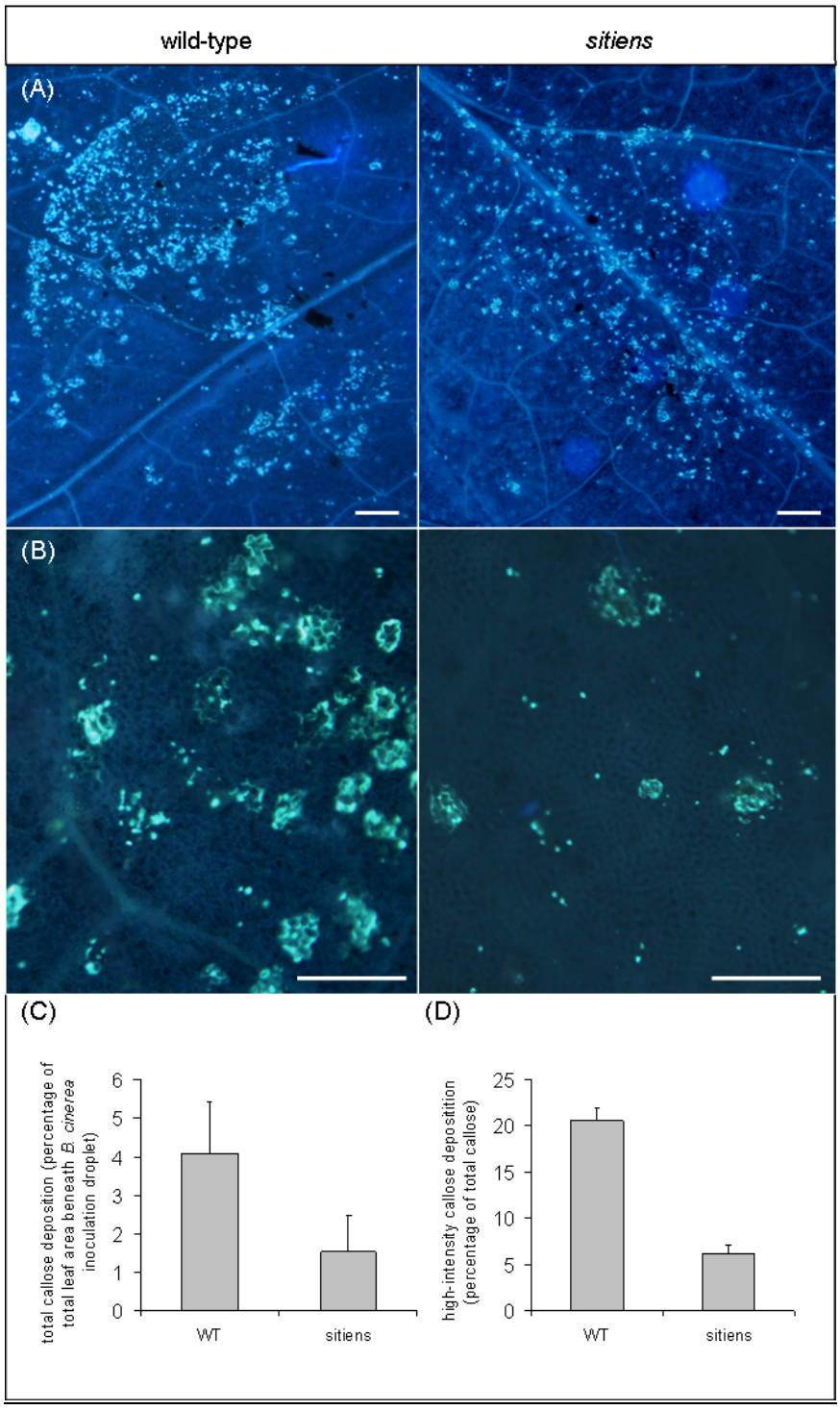

Figure IV-3: Comparison of callose accumulation after *B. cinerea* inoculation in wild-type (WT) and *sitiens* tomato.
(A): Overview-micrographs showing callose deposition in an entire 5 µl *B. cinerea* inoculation drop. (B): Close-up micrograph in a representative part of a *B. cinerea* inoculation drop. (C, D): Quantification of total callose (C) and the proportion of high-intensity callose deposits (D). Micrographs were taken under UV excitation revealing aniline blue-stained callose deposits as bright blue (A) or bright green (B) fluorescence. Representative pictures were selected after staining at least 10 inoculation sites on different *sitiens* and wild-type plants. The values in (C) and (D) were calculated after selection of the total leaf area beneath inoculation droplets, the leaf area covered with "total callose" and the leaf area covered with "high-intensity callose" deposits using the image analysis software package "APS Assess". The values represent the mean and standard error of 10 inoculation sites of different wild-type and *sitiens* plants. The experiment was done three times with similar results. Scale bar = 250 µm.

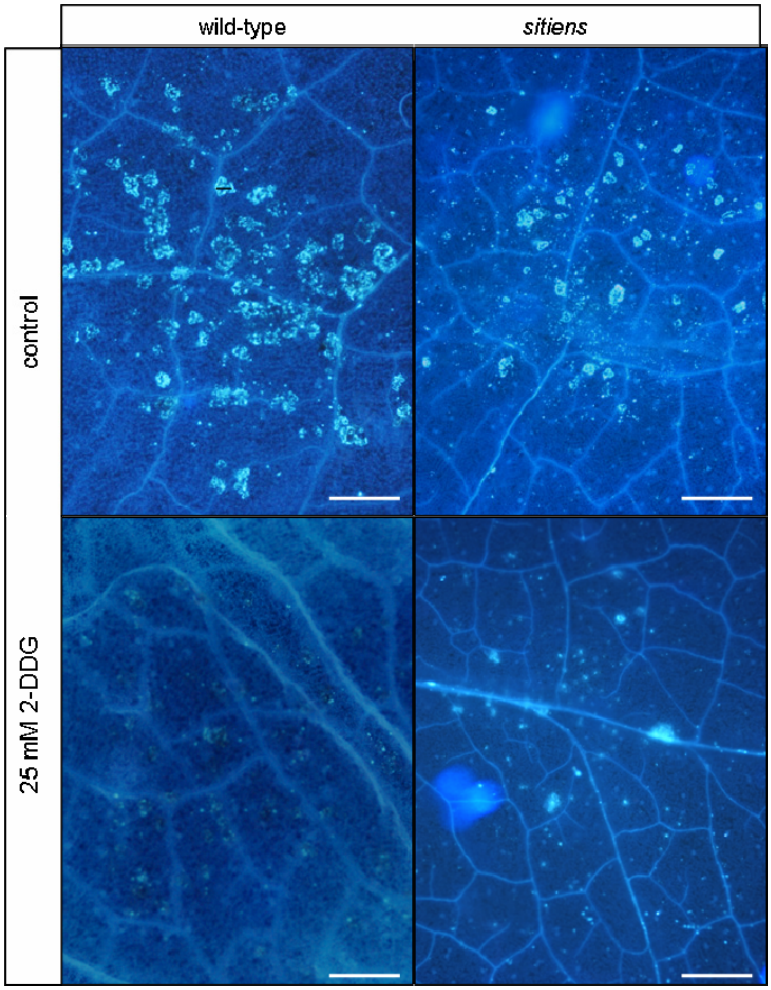

Figure IV-4: Effect of 2-deoxy-D-glucose (2-DDG) on callose accumulation after *B. cinerea* inoculation of wild-type (WT) and *sitiens* tomato.
Micrographs were taken under UV excitation revealing aniline blue-stained callose deposits as bright blue fluorescence. Application of 25 mM 2-DDG removed the *B. cinerea* induced callose accumulation in both *sitiens* and wild type. Representative overview-micrographs showing callose deposition in an entire 5 µl *B. cinerea* inoculation drop were selected after staining at least 10 inoculation sites from different plants for each treatment. The experiment was repeated with similar results. Scale bar = 300 µm.

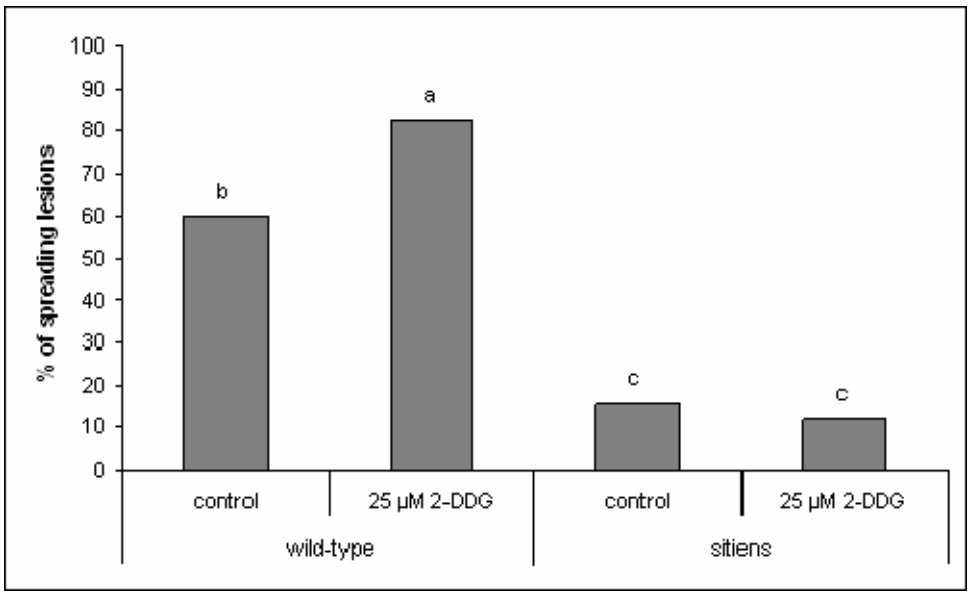

Figure IV-5: Effect of 2-deoxy-D-glucose (2-DDG) on susceptibility of *sitiens* and wild-type tomato to *B. cinerea*.
Application of 2-DDG was done by floating tomato leaf discs in a 25 mM 2-DDG solution 5 h before pathogen inoculation. For each treatment at least 18 leaf discs from at least eight plants were inoculated and the number of spreading lesions was evaluated at 4 days post inoculation. The data were statistically analyzed using binary logistic regression. Bars with different letters are significantly different at $p<0.05$. The experiment was repeated with similar results. When 2-DDG was applied to the petioles and inoculation was done on complete leaves, similar results were also obtained.

DISCUSSION

In the present study, we show that inoculation of tomato leaves with the necrotrophic pathogen *B. cinerea* is followed by rapid formation of callose at the sites of both unsuccessful and successful fungal penetration. ABA-deficient *sitiens* plants showed a reduction in the amount of callose deposition compared to wild type, but inhibition of callose formation with 2-DDG illustrated that there was no correlation between callose deposition and the observed resistance in this mutant. On the other hand, 2-DDG treatment in wild type inhibited callose formation and provoked additional susceptibility. When evaluating 2-DDG inhibitor experiments, care should be taken regarding the specificity of 2-DDG in inhibiting callose deposition, because 2-DDG is widely used as an inhibitor of glycolysis in mammal systems (Woodward and Hudson, 1954; Jain et al., 1985) and disruption of carbon and energy metabolism after 2-DDG treatment have also been reported in plant cells (Klein and Stitt, 1998). Therefore, the possibility exists that increased susceptibility in wild-type plants after 2-DDG treatment is controlled by callose-independent processes. Nevertheless, our data indicate that callose deposition is not important for the high level of resistance in *sitiens*, but suggest that callose formation is involved in basal tomato defence responses during *B. cinerea* infection and is at least partly depending on a functional ABA biosynthetic pathway.

Contrasting roles for ABA in plant defence have been reported. On the one hand, there are many reports on the role of ABA in increased susceptibility. The interaction of ABA with other well-characterised pathogen defence pathways, more specifically the antagonistic relationships of ABA with salicylic acid (SA) or jasmonate/ethylene signalling, have often been proposed as important mechanisms for ABA-induced susceptibility (see Mauch-Mani and Mauch, 2005 and chapter 1 for review). Analysis of wild-type and *sitiens* gene expression upon *B. cinerea* inoculation revealed a hyperinduction of defence responses resulting from ABA-deficiency and showed stronger SA-dependent defence responses in *sitiens* (Asselbergh et al., 2007; see chapter 2), which confirmed our earlier findings (Audenaert et al., 2002a). In concordance, ABA-induced susceptibility to *P. syringae* in Arabidopsis was partly due to suppression of SA production (Mohr and Cahill, 2007). On the other hand, in Arabidopsis an ABA-dependent pathway with a positive effect on defence through enhanced priming for callose was identified (Ton and Mauch-Mani, 2004). Our data present also a role for ABA in callose deposition in tomato upon inoculation with *B. cinerea*, although loss of callose formation has no direct effect on the high level of resistance in *sitiens* to *B. cinerea*. Taken

together, a complex picture has emerged in which ABA acts as a key regulator of different components of pathogen defence, with apparently adverse effects.

Considering the high frequency by which plants are confronted with an enormous variety in abiotic and biotic stresses, it is essential for the plants survival to respond only when necessary, and only with the appropriate, controlled activation of defences. In other words, a need exists for the plant to prioritise certain defence responses upon pathogen attack and consequentially down-regulate other defences. For example, there is multiple antagonistic cross-talk between the salicylic acid-mediated and jasmonate/ethylene-mediated pathways (Thomma et al., 1998; Spoel et al., 2003; Glazebrook, 2005). Similarly, an antagonistic relationship exists between callose formation and activation of the SA signal transduction defence pathway. This was unambiguously shown in *pmr4* mutants of Arabidopsis that are mutated in callose synthase, which resulted in a loss of pathogen-induced callose deposition and resistance to *Erysiphe cichoracearum* by enhanced activation of the SA signalling pathway (Nishimura et al., 2003). Considering this role of callose to limit other defence responses, it cannot be completely excluded that enhanced activation of defence responses in *sitiens* results from reduced callose formation. Nevertheless, our data adds to the growing body of evidence that ABA acts as an important regulator of the overlap of different signalling networks that control disease resistance and abiotic stress tolerance in several plant species.

Adding to the complexity, are the apparent differences between Arabidopsis and tomato in the effect of ABA on pathogen-induced callose deposition. Whereas our data show that lowering endogenous ABA levels in tomato has a significant effect on the amount of callose deposited after infection, it was shown in Arabidopsis that ABA-deficient and ABA-insensitive mutants are not impaired in basal callose deposition upon infection, but are impaired in the priming of its deposition, after inducing resistance with BABA or exogenous ABA treatment (Flors et al., 2005; Ton et al., 2005; Kaliff et al., 2007). Given the lower basal ABA concentrations present in Arabidopsis (< 5 ng ABA g^{-1} FW) (Mohr and Cahill, 2003) compared to those in tomato (> 100 ng ABA g^{-1} FW) (Achuo et al., 2006), it is tempting to speculate that basal ABA levels in tomato are sufficient to create a primed state for callose deposition, which is not present in *sitiens*, whereas in Arabidopsis, BABA or exogenous ABA treatment is needed to provoke priming for callose deposition. Furthermore, it can be hypothesised that in the tomato-*B. cinerea* interaction, callose formation only has a limited effect, whereas resistance by strong SA-dependent responses -as activated in *sitiens*- is much greater. In Arabidopsis, the strong effect of ABA-induced priming for callose deposition and the limited disease phenotypes

of ABA-mutants indicate a general shift towards effective callose-dependent defences at the expense of restricted SA-dependent defences.

MATERIALS AND METHODS

Plant growth, chemical treatment and *B. cinerea* inoculation

Tomato (*Solanum lycopersicum* L., previously *Lycopersicon esculentum* Mill.) *sitiens* mutants (Taylor et al., 1998; Taylor et al., 2000), provided by Prof. M. Koornneef (Wageningen University, The Netherlands) and the corresponding wild-type cultivar Moneymaker were grown in potting compost soil (Substrat 4; Klasmann-Deilmann, Groß Hesepe, Germany) at 22°C. The plants were raised in a growth chamber with 75% relative humidity in a 16 h/8 h light-dark regime.

After 4 to 5 weeks, when seedlings were at the 5th leaf stage, leaf discs were punched out of the tertiary leaves with a 1-cm diameter cork bore and placed floating with the adaxial side up in 24-well plates (VWR, Leuven, Belgium). Each well was filled with 1.5 ml of distilled water or, if indicated, with 1.5 ml of a solution with 25 mM 2-deoxy-D-glucose (2-DDG, Sigma-Aldrich, St. Louis, MO) dissolved in water. In a second approach, complete leaves were inoculated in infection trays and chemical treatment (2-DDG) was applied by petiole feeding as described (Audenaert et al., 2002a).

Conidia of *B. cinerea* strain R16 (Faretra and Pollastro, 1991) were obtained as previously described (Audenaert et al., 2002a). The conidial suspension was centrifuged for 10 min at 10 000x*g*. After removal of the supernatant and re-suspension of the conidia in distilled water, an inoculation suspension was prepared containing $6.25x10^4$ conidia/ml in 16.7 mM KH_2PO_4 and 25 mM glucose. Conidia were pregerminated for 2 h in the inoculation suspension at 22°C. For leaf disc assays, two 5-µl droplets were used to inoculate each tomato leaf disc and ten 5-µl drops per leaf were used for inoculation of complete leaves. Incubation was done at 22°C under dark conditions. Symptoms were evaluated after 4 days. Each inoculation droplet was classified as a spreading lesion (macerated, water-soaked) or non-spreading lesion (necrotic, dry) and the data were analyzed with a binary logistic regression, as described previously (Audenaert et al., 2002; Asselbergh et al., 2007). At least 72 inoculation drops were evaluated for each treatment.

Callose staining

Two different methods were applied to stain callose deposits. Following the first protocol (Ton and Mauch-Mani, 2004), tomato leaves were fixed in 100% ethanol. After washing in 0.1 M phosphate buffer (pH 7.0), leaf segments were incubated for 15 min in the same buffer containing 0.005% calcofluor (Fluorescent Brightener 28; Sigma) and 0.01% aniline blue. Leaf segments were subsequently washed repeatedly in 0.01% aniline blue in 0.1 M phosphate buffer (pH 7.0) and mounted in glycerol.

In a second approach, (adapted from an "in-house protocol" of the Laboratoire Interactions Plantes-Pathogènes, INRA/INA PG, Paris,) leaf segments were first fixed and decoloured by vacuum infiltration in lactophenol for 30 min and subsequently incubated in fresh lactophenol at 65°C for 30 min and at room temperature for at least 12 h. Leaves were washed in 50% ethanol for 5 min, before a 30 min incubation in darkness in 0.01% aniline blue in 150 mM K_2HPO_4 pH 9.5.

Visualisation was done under UV excitation by using either an Olympus BX51 microscope with U-MWU2 filter cube (330-385 nm excitation filter, DM 400 dichroic beam splitter and BA420 long-pass filter) or a Nikon TE2000-E microscope with 330-380 nm excitation filter, 400 nm dichroic beam splitter and 420 nm long-pass filter.

Quantification of callose deposition was performed with "APS Assess" (Image Analysis Software for Disease Quantification of The American Phytopathological Society) on overview pictures (covering complete *B. cinerea* inoculation droplets) captured with fixed microscope and camera settings. "Total callose" and "high intensity callose" deposits were selected by using fixed settings in the HSI colour space model: "total callose" = 120 < Hue (H) < 190 and "high-intensity callose" = 230 < Intensity (I) < 250. At least 10 representative inoculation sites of different wild-type and *sitiens* plants were used to calculate the percentage of leaf area within the inoculation droplet covered with "total callose" and the proportion of "high-intensity callose" deposits (= "high-intensity callose" / "total callose").

ACKNOWLEDGEMENTS

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Chapter

5

Abscisic Acid-Deficiency Leads to Rapid Activation of Tomato Defence Responses Upon Infection with *Erwinia chrysanthemi*

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In addition to the important role of abscisic acid (ABA) in abiotic stress signalling, basal and high ABA-levels appear to have a negative effect on disease resistance. Using the ABA-deficient *sitiens* tomato (*Solanum lycopersicum*) mutant and different application methods of exogenous ABA, we demonstrated the influence of this plant hormone on disease progression of *Erwinia chrysanthemi* (*Dickeya* spp.*)*. This necrotrophic plant pathogenic bacterium is responsible for soft rot disease on many plant species, causing maceration symptoms mainly due to the production and secretion of pectinolytic enzymes. On wild-type tomato cv. Moneymaker *E. chrysanthemi* leaf inoculation resulted in maceration both within and beyond the infiltrated zone of the leaf, but *sitiens* showed a very low occurrence of tissue maceration, which never extended the infiltrated zone. A single ABA treatment prior to inoculation eliminated the effect of pathogen restriction in *sitiens*, while repeated ABA spraying during plant development rendered both wild type and *sitiens* very susceptible. Quantification of *E. chrysanthemi* populations inside the leaf did not reveal differences in bacterial growth between *sitiens* and wild type. *Sitiens* was not more resistant to pectinolytic cell wall degradation, but upon pathogen inoculation it showed a faster and stronger activation of defence responses than wild type, such as hydrogen peroxide accumulation, peroxidase activation and cell wall fortifications. Moreover, the rapid activation of *sitiens* peroxidases was also observed after application of bacteria-free culture filtrate containing *E. chrysanthemi* cell wall degrading enzymes and was absent during inoculation with an *out E. chrysanthemi* mutant impaired in secretion of these extracellular enzymes.

INTRODUCTION

The plant hormone abscisic acid (ABA) not only plays an important role in plant development and responses to abiotic stresses (Fujita et al., 2006), but is also involved in biotic stress signalling (Mauch-Mani and Mauch, 2005). In general, ABA appears to suppress basal plant resistance to both biotrophic and necrotrophic fungi and bacteria. For example, the ABA-deficient *aba2-1* mutant of *Arabidopsis* was more resistant to *Fusarium oxysporum* than the wild-type plant (Anderson et al., 2004), while the *aba1-1* mutant was less susceptible to *Hyaloperonospora* (formally *Peronospora*) *parasitica* (Mohr and Cahill, 2003). The ABA-deficient *sitiens* mutant of tomato (*Solanum lycopersicum)* was more resistant than the wild type Moneymaker to *Botrytis cinerea* (Audenaert et al., 2002a; see also chapter 2), *Oidium neolycopersici* (Achuo et al., 2006) and *Pseudomonas syringae* (Thaler and Bostock 2004). Thus, in various plant-pathosystems, low ABA levels seem to lower disease susceptibility, while exogenous ABA application enhances pathogen susceptibility. However, positive effects of ABA on pathogen defence have also been observed (see chapter 1 or Mauch-Mani and Mauch, 2005 for review). The question arises as to how leaf ABA content influences the plant's susceptibility to pathogens.

During plant-pathogen interactions, ABA interacts in various ways with other plant hormone signalling pathways, mediated by salicylic acid (SA), jasmonate (JA) and ethylene (ET). There is increasing evidence of important overlap between ABA regulatory networks and the pathways of plant response to pathogens, with ABA interfering at different levels of biotic stress signalling, depending on the plant-pathosystem (see chapter 1 or Mauch-Mani and Mauch, 2005 for review). Very recent studies in the *Arabidopsis*-*Pseudomonas syringae* pv. *tomato* interaction revealed that ABA induces susceptibility through suppression of SA accumulation and cell wall fortification (Mohr and Cahill, 2007). Moreover, bacterial type III-secreted effectors were shown to manipulate ABA biosynthesis and ABA signalling as a virulence strategy to suppress defence responses (de Torres-Zabala et al., 2007).

During abiotic stress, ABA-derived signal transduction often involves accumulation of hydrogen peroxide (H_2O_2) (Pei et al., 2000; Apel and Hirt, 2004). On the other hand, in physiological processes such as seed germination, the release of H_2O_2 during seed germination was inhibited by ABA (Schopfer et al., 2001). The production of high amounts of H_2O_2 and other reactive oxygen species (ROS) during pathogen attack, referred to as the oxidative burst, is an essential and well-studied element in plant defence that can have a direct antimicrobial

function or can have a function in defence signalling, the hypersensitive response and cell wall strengthening (Lamb and Dixon, 1997; Apel and Hirt, 2004). However, whether ABA can influence ROS accumulation during pathogen defence remains unknown.

The necrotrophic bacterium *Erwinia chrysanthemi* (now reclassified in *Dickeya* species by Samson et al. (2005)) causes soft rot on storage organs, succulent stems and leaf tissue of a wide range of greenhouse and open field-grown plant species of diverse botanical families, including several *Solanaceae* species (Schaad and Brenner, 1977; Dickey, 1979; Pérombelon and Kelman, 1980). On greenhouse-cultivated tomato, *E. chrysanthemi* causes stem rot by systemic infection from the roots and rapidly induces wilt and eventual collapse of the whole plant (Aysan et al., 2003). The virulence of *E. chrysanthemi* is largely attributed to its ability to secrete plant cell wall degrading enzymes (CWDEs) and particularly pectinases (Collmer and Keen, 1986; Kotoujansky, 1987). Most *E. chrysanthemi* CWDEs are secreted to the external medium via a common type II secretion system, the Out machinery, with Out-negative mutants being unable to efficiently degrade plant cell walls (Andro et al., 1984). Induction of the secretion machinery-encoding genes and the production of exo-enzymes and other virulence factors is tightly regulated and mediated by a number of global regulatory circuits, such as the *KdgR* regulon and the *PecS-PecM* regulatory couple. Environmental stimuli that were identified to regulate the synthesis of virulence factors are pectin-degradation intermediates and other inducers from plant extracts, but also the plant's redox-status (reviewed in Barras et al., 1994; Hugouvieux-Cotte-Pattat et al., 1996; Expert, 1999).

In the present study, we analysed the role of ABA in the tomato – *E. chrysanthemi* interaction using the ABA-deficient *sitiens* mutant and exogenous ABA feeding. *Sitiens* appeared to be very resistant to *E. chrysanthemi* and the nature of this resistance was studied more in detail by testing the following hypotheses: (1) *sitiens* resistance is due to morphological differences; (2) *sitiens* resistance is due to inhibition of bacterial growth; (3) *E. chrysanthemi* CWDEs are ineffective in degrading *sitiens* cell walls; (4) active defence responses are triggered earlier or more efficiently in *sitiens*.

RESULTS

Disease symptoms in the *E. chrysanthemi*-tomato interaction

When *E. chrysanthemi* was infiltrated into leaf tissue of tomato cv. Moneymaker, the first symptoms could already be seen within 8 hours post inoculation (hpi) and complete maceration of the infiltrated zone, consisting of wet and degraded leaf tissue, occurred within 24 hpi (Fig. V-1a). In some cases, maceration of the infiltrated tissue aborted and a necrosis developed (Fig. V-1b). A spreading maceration usually progressed into non-infiltrated parts of the inoculated leaflet (Fig. V-1c), often extending into non-inoculated leaflets on the same leaf (Fig. V-1d), and eventually through the primary petiole into the stem, causing lodging of the plant (Fig. V-1e).

In addition to local symptoms, *E. chrysanthemi* caused systemic symptoms, visible within 7 days post inoculation (dpi). These systemic symptoms include maceration of leaves above the inoculated leaf without obvious stem maceration (Fig. V-1g), shoot apex death (Fig. V-f) or total collapse of the plant.

ABA deficiency in the *sitiens* mutant of tomato results in resistance to *E. chrysanthemi*.

To investigate the effect of endogenous ABA on tomato susceptibility to *E. chrysanthemi* infection, the ABA-deficient *sitiens* mutant was compared to its wild-type parent cv. Moneymaker. Inoculation of greenhouse-grown plants with a concentration of 10^7 CFU/ml resulted in maceration of more than half of wild-type inoculation sites within 24 hpi, of which about 20% exhibited spreading maceration during the following days. In contrast, only 10% of inoculated *sitiens* leaves showed maceration of the infiltrated tissue, and spreading of maceration outside the infiltrated zone was never observed (data not shown).

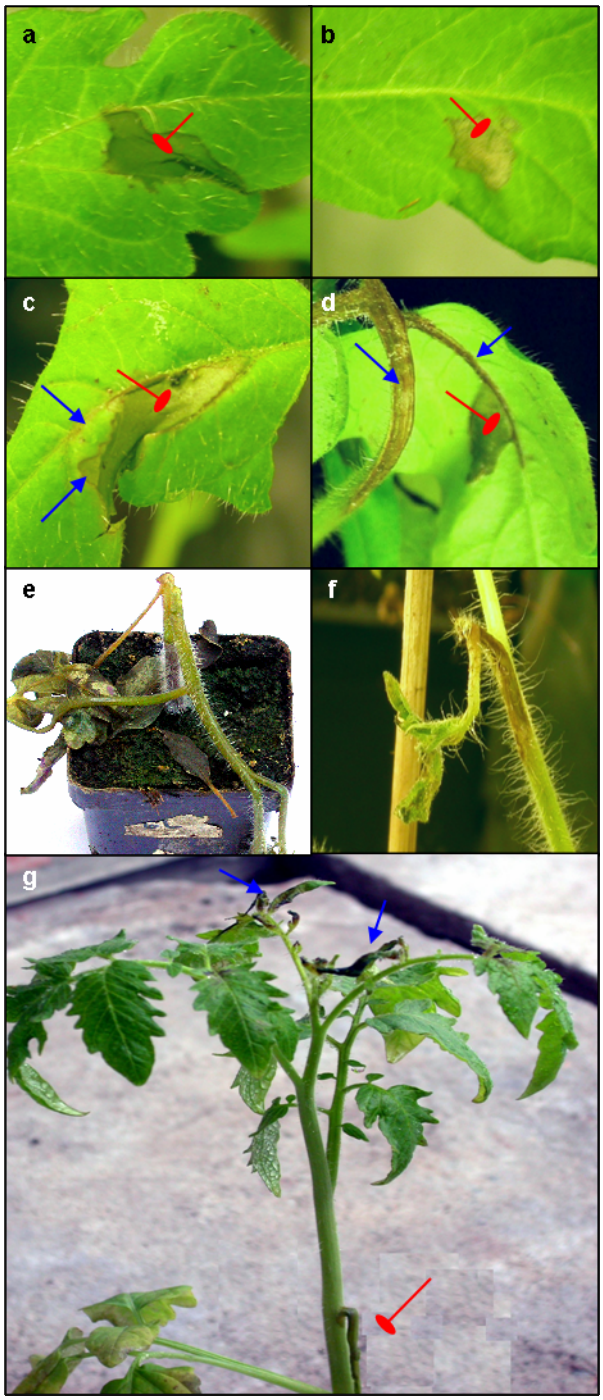

Figure V-1: Disease symptoms on tomato cv. Moneymaker caused by syringe infiltration of 10^7 CFU *E. chrysanthemi*/ml. (a): maceration contained in the infiltrated zone (24 hpi); (b): necrosis of the infiltrated zone (24 hpi); (c): maceration spreading beyond the infiltrated zone (sharp arrows) (48 hpi); (d): maceration spreading to petiolule and petiole of inoculated leaf (sharp arrows) (72 hpi); (e): complete collapse of plant due to stem maceration (7 dpi); (f): death of shoot apex due to systemic infection (7 dpi); (g) spread of local maceration to base of petiole of inoculated leaf (blunt arrow) and maceration of systemic leaves (sharp arrows) (7 dpi). Blunt arrows in (a), (b), (c) and (d) indicate the infiltrated zone.

As ABA affects numerous plant developmental and physiological processes, including water balance and flux, the *sitiens* tomato mutant exhibits a different morphology compared to the wild type (Nagel et al., 1994; see supplementary figure V-S1). To determine if these structural changes in the plant phenotype could be completely or partly responsible for *sitiens*' loss of susceptibility to *E. chrysanthemi*, an inoculation was done on plants raised in a growth chamber at 28°C and 100% relative humidity (RH); conditions that eliminate visible morphological differences between the two genotypes.

Two different bacterial inoculum concentrations were tested. Leaf infiltration with 10^6

CFU/ml caused local maceration in more than 50% of wild-type leaves within 48 hpi, while only a few *sitiens* leaves exhibited local maceration that aborted within the next 24 hours (Fig. V-2a). Such aborted maceration was also observed in a few cases on wild-type leaves at 72 hpi, but more often maceration was spreading, indicating an overall disease progression in wild-type plants. Leaf infiltration with a more aggressive inoculum (10^7 CFU/ml) resulted in a relatively higher number of macerated leaves in general, but *sitiens* plants were still remarkably healthy compared to wild-type plants and no maceration was observed beyond the infiltration site (Fig. V-2a). Furthermore, at 7 dpi the aggressive inoculum did not cause systemic infection of *sitiens* whereas in the wild type, systemic infection occurred in more than half of the plants resulting in complete collapse of some plants (data not shown).

Exogenous ABA increases susceptibility of tomato to *E. chrysanthemi*

To further confirm the role of ABA in the susceptibility of tomato to *E. chrysanthemi*, both wild-type and *sitiens* plants were supplied with exogenous ABA by supplementary feeding during plant growth or by pulse feeding four hours before challenge with the pathogen. Whereas supplementation in *sitiens* increases ABA content and restores the wild-type leaf morphology (Achuo et al., 2006), pulse treatment is expected to increase leaf ABA content without changing the *sitiens* phenotype. To investigate the effect of leaf structural modification in *sitiens*, these experiments were conducted on plants grown in a greenhouse at relatively low temperatures (21±1/18±1°C day/night) and RH (±65%), to permit expression of the typical *sitiens* leaf morphology and of the ABA supplementation effect on structural modification (see supplementary figure V-S1). Incubation after bacterial inoculation was done at high RH (±100%), as in previous experiments. Both ABA application by pulse treatment and by supplementation resulted in a significant loss of *sitiens* resistance to *E. chrysanthemi* infection (Fig. V-2b). Pulse treatment with ABA restored the wild-type level of susceptibility in *sitiens*, while supplementation resulted in significantly higher susceptibility than untreated wild-type plants. Exogenous ABA also increased the susceptibility of wild-type plants, causing maceration in all inoculation sites compared to about 2/3 of inoculation sites in the control at 24 hpi. At 48 hpi, nearly all inoculation sites of ABA supplemented wild-type plants exhibited spreading maceration symptoms, compared to about 30% of spreading maceration that was observed in pulse-treated inoculation sites (Fig. V-2b).

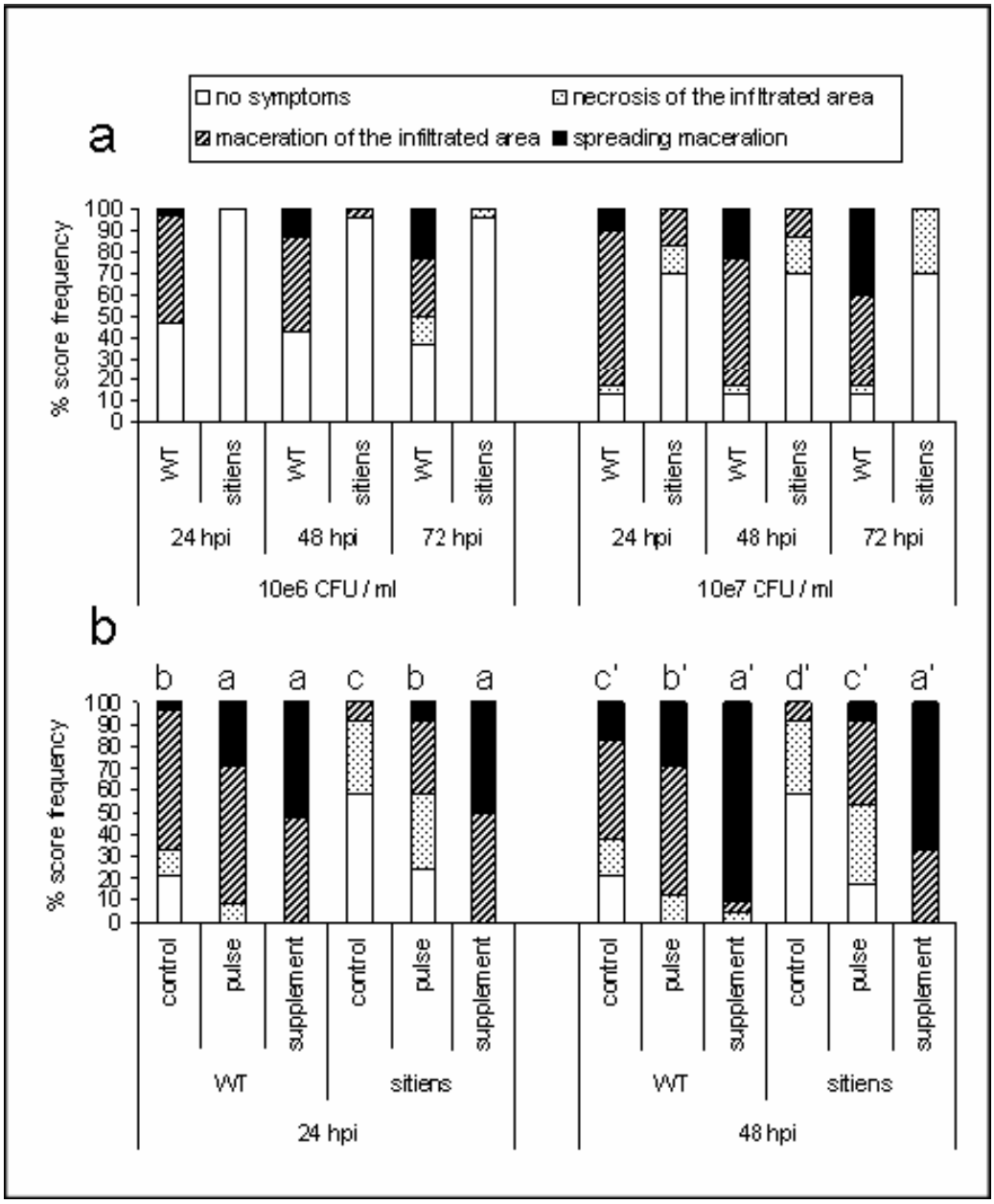

Figure V-2: Effect of abscisic acid (ABA) on *E. chrysanthemi* disease symptoms on tomato.
(a): Disease progression at 24, 48 and 72 hpi on *sitiens* and wild-type (WT) tomato, grown under conditions of high temperature (28°C) and high relative humidity (100%), and infiltrated with 10^6 or 10^7 CFU *E. chrysanthemi*/ml. At all time points and for both inoculum concentrations, disease development was significantly lower in *sitiens* than in the wild-type parent at $p = 0.05$.
(b): Effect of different exogenous ABA treatments on *sitiens* and wild-type susceptibility to *E. chrysanthemi*. Plants were sprayed with 100 µM ABA at 3-4 day intervals for two weeks (physiological supplementation - "supplement" treatment) or 4 hours before inoculation ("pulse" treatment) and infiltrated with 10^7 CFU/ml. Bars with different letters indicate a significant difference between the treatments at $p = 0.05$.
Disease development was evaluated and data analysed by the Kruskal-Wallis/Mann-Whitney nonparametric test. At least 8 plants per genotype-treatment combination were used and on each plant, disease was evaluated on three infiltrated leaflets of the same leaf. For (a) and for (b), similar results were obtained in at least three independent experiments. Data of one representative experiment are presented.

E. chrysanthemi in planta multiplication and effect of cell wall degrading enzymes on *sitiens* and wild-type leaf tissue

To check whether the resistance of *sitiens* could be due to inhibition of bacterial growth, inoculated leaf material was sampled at different time points and plated out on LB medium to monitor local bacterial growth in the infiltrated tissue. Since signs of maceration became macroscopically visible at 24 hpi, samples at this time point were taken separately from macerating and non-macerating infiltrated tissue of *sitiens* and wild-type leaves. The same amount of bacteria was retrieved in both wild-type and *sitiens* leaves just after inoculation (0 hpi), showing that equal amounts of bacteria per square mm were infiltrated in both genotypes (Fig. V-3). Within 12 hours after infiltration the bacteria grew at a similar rate and to the same extent in wild-type and in *sitiens* leaves. Bacterial populations stabilised in non-macerated tissue of both *sitiens* and wild type at 12 hpi, while the bacteria continued to grow in macerated tissue of both genotypes (Fig. V-3). While sampling at 48 hpi was not possible for macerated leaves due to advanced disintegration of the infiltrated tissue, the bacteria population in non-macerated infiltrated tissue at 48 hpi was similar to that at 24 hpi (data not shown).

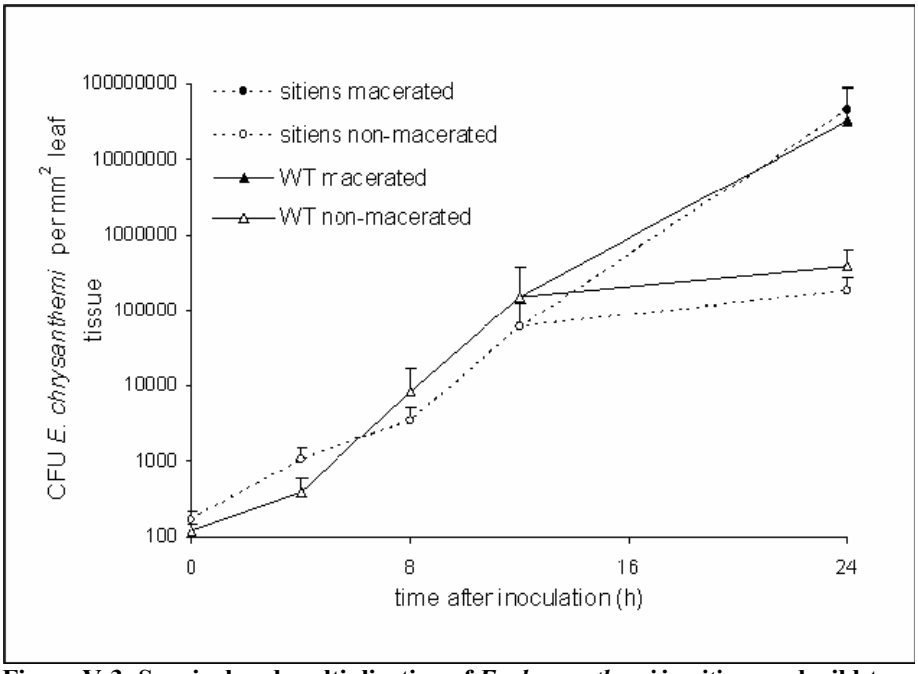

Figure V-3: Survival and multiplication of *E. chrysanthemi* in *sitiens* and wild-type (WT) tomato leaf tissue. Leaf discs were taken from infiltrated tissue at the indicated time points, crushed in 50 mM KCl, plated out on LB medium and incubated at 28 °C for 24 hours. Colonies of *Erwinia chrysanthemi gfp9* strain were identified under UV-light by their green fluorescence and counted. Bacterial counts at each time point were compared statistically by the t-test and no significant differences were detected between *sitiens* and wild type before 24 hpi in three independent experiments. Each data point represents the means + the standard error from 1 experiment using 5 or 6 plants per treatment. At 24 hpi, 5 macerated and 5 non-macerated samples from wild type and from *sitiens* were selected, without representing the frequency of maceration in both genotypes.

To test whether *sitiens* cell walls are more resistant to cell wall degrading enzymes (CWDEs) than wild-type cell walls, various dilutions of a bacterial culture filtrate (CF) containing CWDEs were infiltrated in *sitiens* and wild-type leaves. When undiluted CF was infiltrated, cell wall degradation could already be observed within 1 hpi on both genotypes. This was seen macroscopically as a soft and transparent appearance of the infiltrated tissue, resembling maceration symptoms caused by the pathogen, and confirmed microscopically by the absence of cell walls and a protoplast-like appearance of mesophyll cells (data not shown). The kinetics of occurrence of maceration symptoms on wild type and *sitiens* was followed after infiltration of CF serial dilutions in detached leaves. The time at which one half of the leaves presented signs of maceration was extrapolated for each CF dilution and plotted against the dilution factor. The kinetics of macroscopically visible cell wall degradation occurrence was similar in both genotypes, and delayed upon dilution of the CF (see supplementary figure V-S2).

These findings oppose the hypotheses that *sitiens* resistance is caused by preventing *E. chrysanthemi* to multiply, or by ineffectiveness of *E. chrysanthemi* CWDEs to degrade *sitiens* cell walls.

Hydrogen peroxide accumulation, peroxidase activity and cell wall fortification in wild type and *sitiens* during infection with *E. chrysanthemi*

Since early and strong hydrogen peroxide (H_2O_2) accumulation is often observed in active defence responses, we studied this reaction in wild-type and *sitiens* tomato leaves infiltrated with *E. chrysanthemi* by using the DAB staining technique described by Thordal-Christensen et al. (1997). Accumulation of H_2O_2, visualised as brown precipitations formed by oxidation of DAB, was detectable in leaves infiltrated with *E. chrysanthemi*, but not in buffer-infiltrated (mock) leaves. In wild-type leaf tissue, H_2O_2 accumulation was first detected in the infiltrated zone 24 hpi. If maceration was spreading outside the infiltrated area at 48 hpi, H_2O_2 accumulation was located here as well. In *sitiens*, H_2O_2 already accumulated weakly at 12 hpi, followed by a more intense staining at 24 and 48 hpi (see supplementary figure V-S3). A remarkable difference in H_2O_2 accumulation between *sitiens* and wild type was found at the border of the infiltrated zone: while wild type displayed a more diffuse and gradual decline of DAB staining towards the outside of the lesion, the borders of *sitiens* lesions were sharp and intensely stained. This observation was confirmed by microscopical examination of the lesion

borders on intact leaf discs (Fig. V-4a) and on cross-sections of leaf tissue that were embedded after DAB staining (Figures V-4b, 4c and 4d). Within the *E. chrysanthemi* infiltrated zone of both wild-type and *sitiens*, H_2O_2 was predominantly situated in the chloroplasts of mesophyll cells. Wild-type lesion margins showed a gradual decrease in H_2O_2 staining intensity towards the outer part of the lesion, while there was an abrupt stop of DAB-positive mesophyll cells in *sitiens* (Figures V-4a and 4b). Interestingly, in all samples that were examined, in addition to the H_2O_2 accumulation in chloroplasts, the lesion borders in *sitiens* were located at sites of intense extracellular H_2O_2 staining, originating from cells close to minor veins. In these regions with high cellular density and without large intercellular spaces, DAB accumulated in the walls of mesophyll, epidermal and bundle sheath cells (Figures V-4b and 4c). Moreover, by toluidine blue staining after sectioning of bacteria, chloroplasts and cell walls, we could observe that in *sitiens* the regions with apoplastic H_2O_2 delineate restriction of bacterial progression (Fig. V-4d). In infected wild-type leaves no extracellular H_2O_2 accumulation was present and tissue regions with vascular bundles did not form barriers for bacterial progression (Fig. V-4d). At 48 hpi, extracellular H_2O_2 accumulation was further amplified at the borders of *sitiens* lesions, leading to several layers of intensely-stained cells adjacent to the infiltrated zone, which was by now severely necrotized or macerated (data not shown).

We further examined the activity of peroxidases, enzymes important in many H_2O_2-dependent pathogen defence processes. Leaf discs containing infiltrated zones were sampled and peroxidase activity was measured with the tetramethylbenzoidine (TMB) assay described by Ros Barceló (1998). Elevated levels of peroxidases were detected in *sitiens* leaf discs as early as 8 hpi, but this was delayed in wild type until 24 hpi and the levels remained significantly lower than for *sitiens* (Fig. V-5a). Buffer-infiltrated and non-infiltrated control samples also showed a limited increase in peroxidase activity, probably due to the effect of syringe infiltration and exposure to inoculation conditions, respectively. Interestingly, activities in control samples of *sitiens* increased earlier and reached slightly higher levels than wild-type controls, as was the case in *E. chrysanthemi* infiltrated samples. Nevertheless, activity values in the respective controls of both wild type and *sitiens* always remained significantly lower than in pathogen-inoculated samples (Fig. V-5a). Pulse application of exogenous ABA did not alter the early peroxidase activation at 8 hpi in *sitiens*, but significantly reduced peroxidase activity during the later stages of inoculation (Fig. V-5b). A concentration of 100 µM ABA was sufficient to decrease peroxidase activity to wild-type levels. Exogenous ABA had no significant effect on peroxidase activity in wild type.

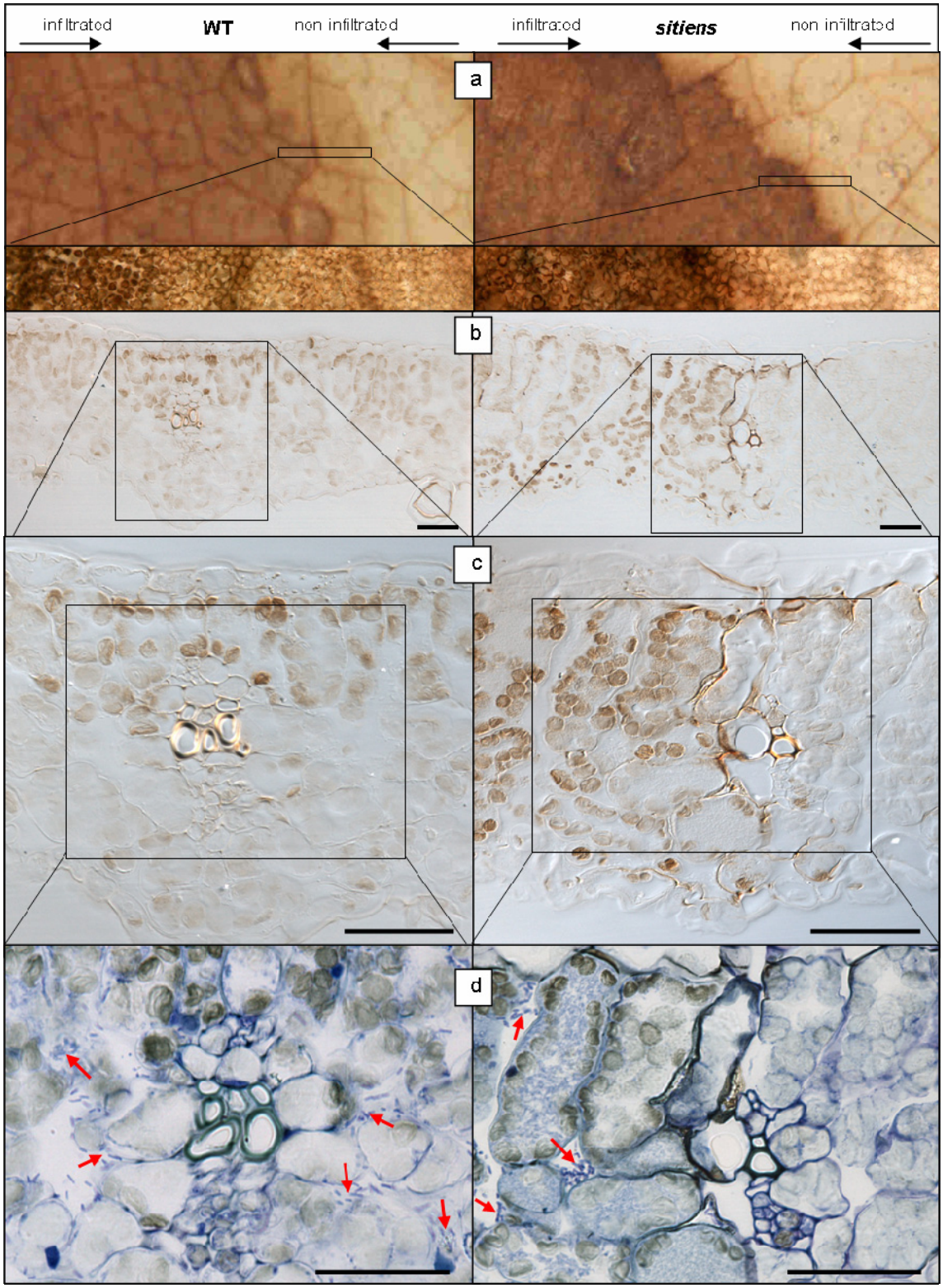

Figure V-4: H_2O_2 accumulation at the border of *E. chrysanthemi* lesions in wild-type (WT) and *sitiens* leaf tissue 24 hpi.

DAB accumulation was evaluated on intact leaf discs (a), and on cross-sections without (b and c) and with (d) toluidine blue staining. Lesions in wild type are characterised by a gradual decrease in chloroplastic H_2O_2 accumulation towards the outside of the lesion, while *sitiens* lesions have a clear border between mesophyll cells that contain or lack chloroplastic H_2O_2. This border in *sitiens* is located at the site of minor veins and in addition, cells in the vicinity of vascular tissues of the border accumulate extracellular H_2O_2. Toluidine blue staining (d) reveals bacteria on both sides of the minor vein in wild-type leaves, while in *sitiens* no bacteria are present beyond the zone of extracellular H_2O_2 accumulation. Some bacterial micro-colonies are marked with arrows.
Scale bar = 50 µm.

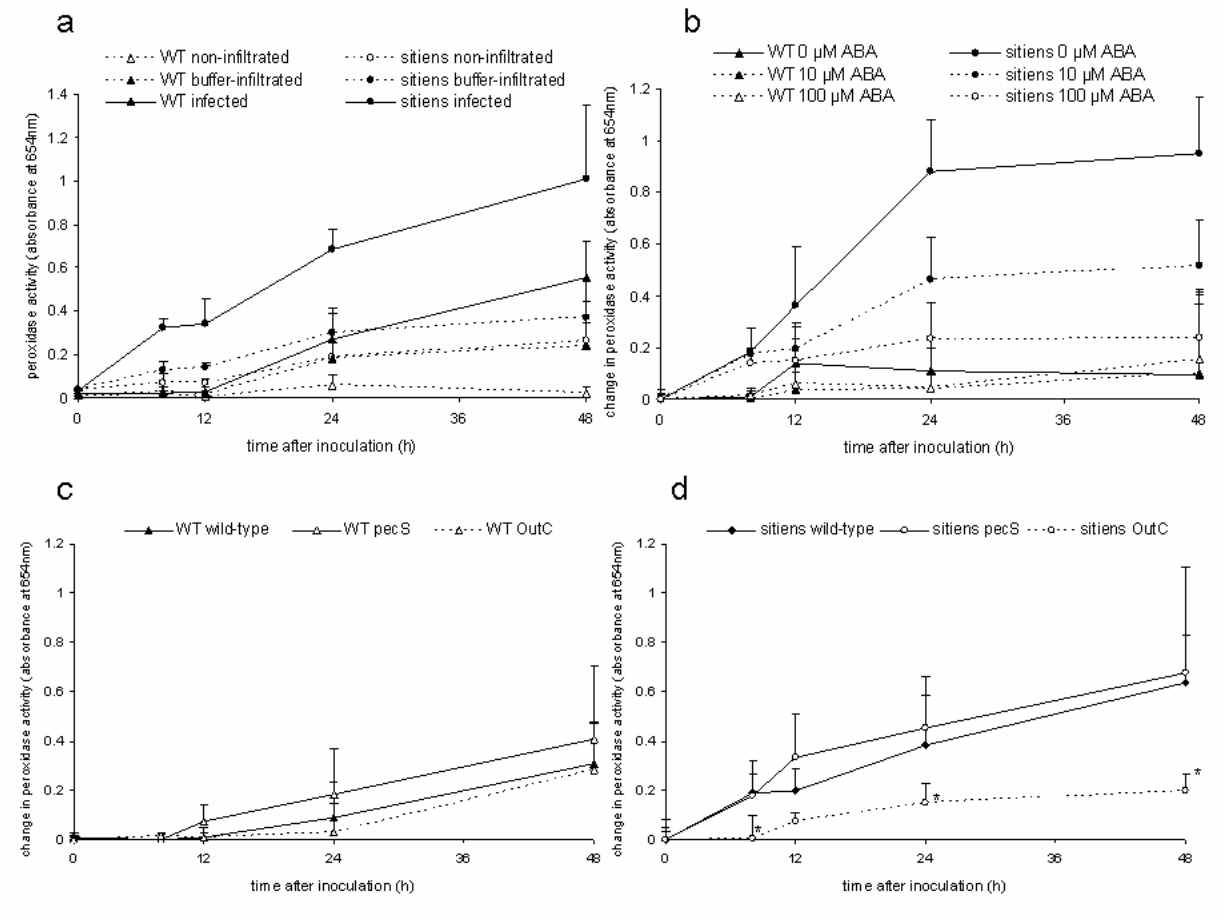

Figure V-5: Extracellular peroxidase activity in wild type and *sitiens* inoculated with *E. chrysanthemi*.
(a): Leaf discs were sampled from leaves infiltrated with 10^6 CFU/ml *E. chrysanthemi* ("infected") or 50mM KCl ("buffer-infiltrated"). Leaf discs without infiltration zone were also sampled ("non-infiltrated").
(b): Leaf discs were sampled from wild type and *sitiens* infiltrated with 10^6 CFU/ml *E. chrysanthemi* four hours after a single spray with 0, 10 or 100 µM ABA. All spray solutions contained 0.05% ethanol.
(c, d): Leaf discs were sampled from wild-type (c) and *sitiens* (d) leaves infiltrated with 10^6 CFU/ml of *E. chrysanthemi* wild-type strain, the pathogenicity factor overproducer *pecS* strain and the type II secretion-deficient *outC* strain. In (b), (c) and (d), the presented change in peroxidase activity is the absolute activity value after subtraction of the activity level of buffer-infiltrated samples for each time point. During sampling, discs were fixed in ethanol and peroxidase activity was measured at 654 nm after addition of TMB and 0.03% H_2O_2. The mean + standard error of the absorbance of the incubation solution from four discs of different plants are presented. In (c) and (d), significant differences at p=0.05 between wild-type and the two mutant treatments were determined by using one-way ANOVA and Duncan post-hoc tests for each time point and are indicated with "*". All experiments were repeated with similar results.

An important consequence of the activation of peroxidases and the production of extracellular H_2O_2 upon pathogen attack is the fortification of the plant cell wall by peroxidative incorporation of phenolic compounds and cross-linking of cell wall proteins (Bradley et al., 1992; Passardi et al., 2005). We stained phenolic compounds in fortified cell walls with safranin-o, and cross-linked proteins were visualised with Coomassie blue staining after SDS denaturation (Fig. V-6). In *sitiens*, cell wall fortification was observed at the site of extracellular H_2O_2 accumulation, i.e. the border of the infiltrated zone, where both stains accumulated strongly. In wild-type tomato, cell wall fortification staining was absent or weak. Both genotypes showed an additional weak intracellular accumulation of the stains in the zones of the

leaf infiltrated with *E. chrysanthemi*, representing dead cells stained after influx through damaged cell membranes and non-specific binding of the staining molecules (Fig. V-6).

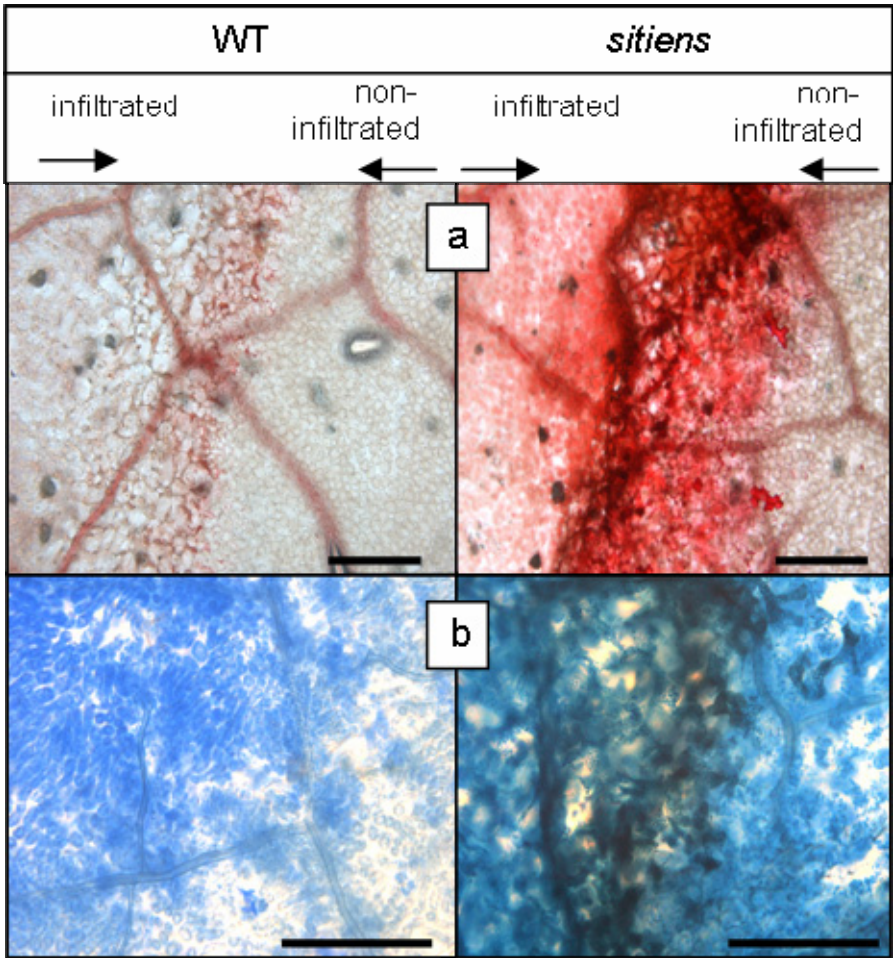

Figure V-6: Cell wall fortification in wild-type (WT) and *sitiens* tomato 48 h after inoculation with *E. chrysanthemi*. Cell wall fortifications were visualized with safranin-o (red-pink) to detect incorporation of phenolics (a) and Coomassie blue staining after SDS denaturation to detect protein cross-linking (dark blue) (b). In both genotypes, a weak accumulation of the stains is present inside dead cells of the infiltrated zone (left), while the non-infiltrated zone (right) remains unstained. Cell wall fortifications at the border of the infiltrated zone in *sitiens* are strongly stained. Representative borders of *sitiens* and wild type were selected after observation of at least ten inoculation sites. The experiment was repeated with similar results. Scale bar = 200 µm.

Effect of *E. chrysanthemi* pectinolytic cell wall degradation on peroxidase activity in wild type and *sitiens*

To determine whether CWDEs play a role in activation of the observed active plant defence response in *sitiens*, we used mutant strains of *E. chrysanthemi* altered in the production of CWDEs to infiltrate wild-type and *sitiens* leaves. *E. chrysanthemi pecS* strain is mutated in the transcriptional repressor gene *pecS*, a mutation leading to derepressed synthesis of pectinases, cellulases and secretion-machinery proteins and overproduction of indigoidine, a blue pigment involved in pathogenicity and in resistance to oxidative stress (Reverchon et al., 1994); *outC* mutant strain is affected in type II secretion and is thereby incapable of secreting several proteins, mainly pectinolytic enzymes and cellulase (Andro et al., 1984; Kazemi-Pour et al., 2004). As expected, infiltration of tomato leaves with *outC* did not cause tissue maceration, but on some plants the infiltrated zone showed necrosis (Fig. V-7). Symptom development after

pecS infiltration was not significantly different from that after wild-type *E. chrysanthemi* infiltration on both wild type and *sitiens*. However, in *sitiens* at 24 hpi, *pecS* was able to cause maceration on a few plants while wild-type *E. chrysanthemi* could not. Nevertheless, by 48 hpi all disease progression had ceased in *sitiens* and no spreading maceration was observed (Fig. V-7). Bacterial quantification in the infiltrated zone did not reveal differences between the mutant strains in the ability to grow inside wild-type and *sitiens* leaf tissue between 0 and 12 hpi (see supplementary figure V-S4). In wild-type leaves, no differences in peroxidase activity were detected after inoculation with the three *E. chrysanthemi* strains (Figure V-5c). In *sitiens*, derepression of pathogenicity factor production in the *pecS* strain did not cause a significant change in peroxidase activity either when compared with wild-type *E. chrysanthemi*. However, the *outC* secretion mutant caused delayed and lower peroxidase activation than the wild-type strain (Fig. V-5d).

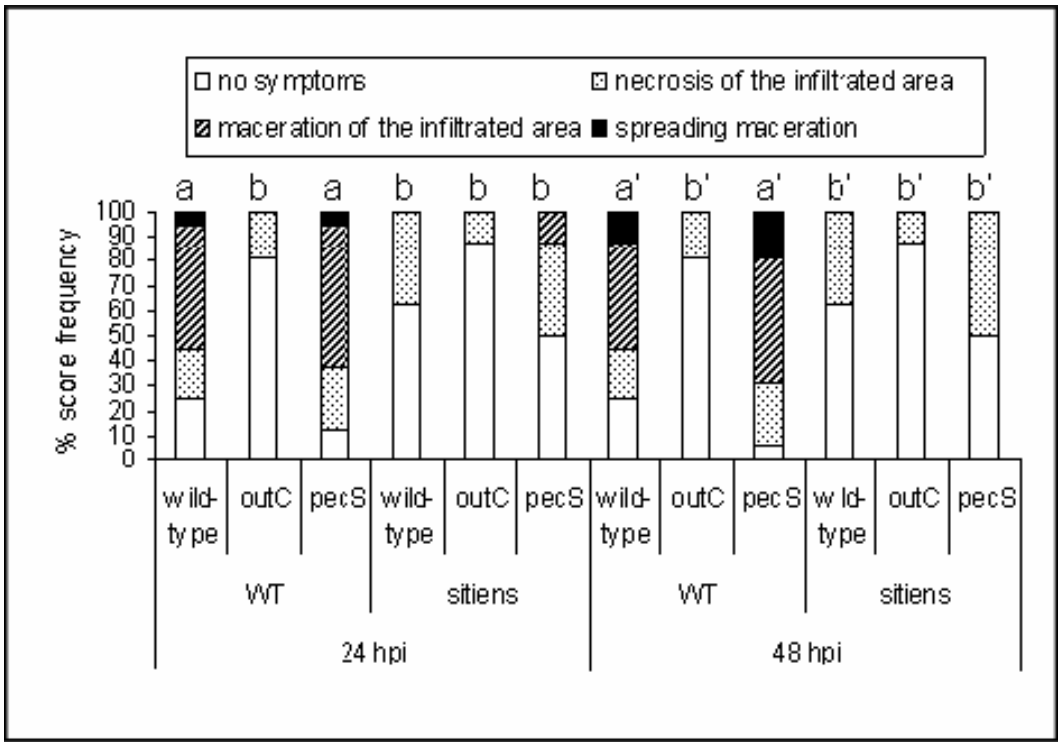

Figure V-7: Disease progression of *E. chrysanthemi* strains with different pectinase production on *sitiens* and wild-type (WT) tomato at 24 and 48 hpi.
The 2nd youngest leaf of plants at 3-4 leaves-stage were infiltrated with 10^6 CFU/ml of *E. chrysanthemi* wild type strain, the pathogenicity factor overproducer *pecS* strain and the type II secretion-deficient *outC* strain. Disease development was evaluated at 24 and 48 hpi and data analysed by the Kruskal-Wallis/Mann-Whitney nonparametric test. Bars with different letters indicate a significant difference between the treatments at $p = 0.05$. Similar results were obtained from two independent experiments using at least 8 plants per treatment. Data from 1 experiment are presented.

These results show that in wild-type tomato, the presence of *E. chrysanthemi* CWDEs does not significantly raise peroxidase activity levels, while in *sitiens*, type II secretion is necessary for the activation of peroxidases. To confirm a role for Out-secreted proteins in

sitiens defence activation we measured peroxidase activity after application of bacteria-free *E. chrysanthemi* supernatants of bacterial cultures grown on a pectinase-inducing medium. Pectinase production was triggered on a medium containing polygalacturonic acid (PGA) as described above, and the CF dilution that was used (0.3) was sufficient to cause macroscopically visible tissue maceration within 5 hpi (see also supplementary figure V-S2). Peroxidase activity increased strongly in *sitiens* within 5 hours after CF application (Fig. V-8a). In wild-type leaves or *sitiens* leaves that were treated with ABA, CF infiltration did not result in an increase of peroxidase activity that was significantly different from the respective control treatments at any of the examined time points (Fig. V-8a, 8b and 8c). If pectinase-free *outC* CF was applied, the activity increase in *sitiens* was limited and did not exceed the levels of the control treatment containing pure medium (Fig. V-8d). In addition, heat treatment of the CF (15 min at 80°C) resulted in a loss of the CF's ability to cause a strong increase in *sitiens* peroxidase activity, which indicates that denaturation of type II secreted proteins in the CF results in a failure to trigger *sitiens* defence responses.

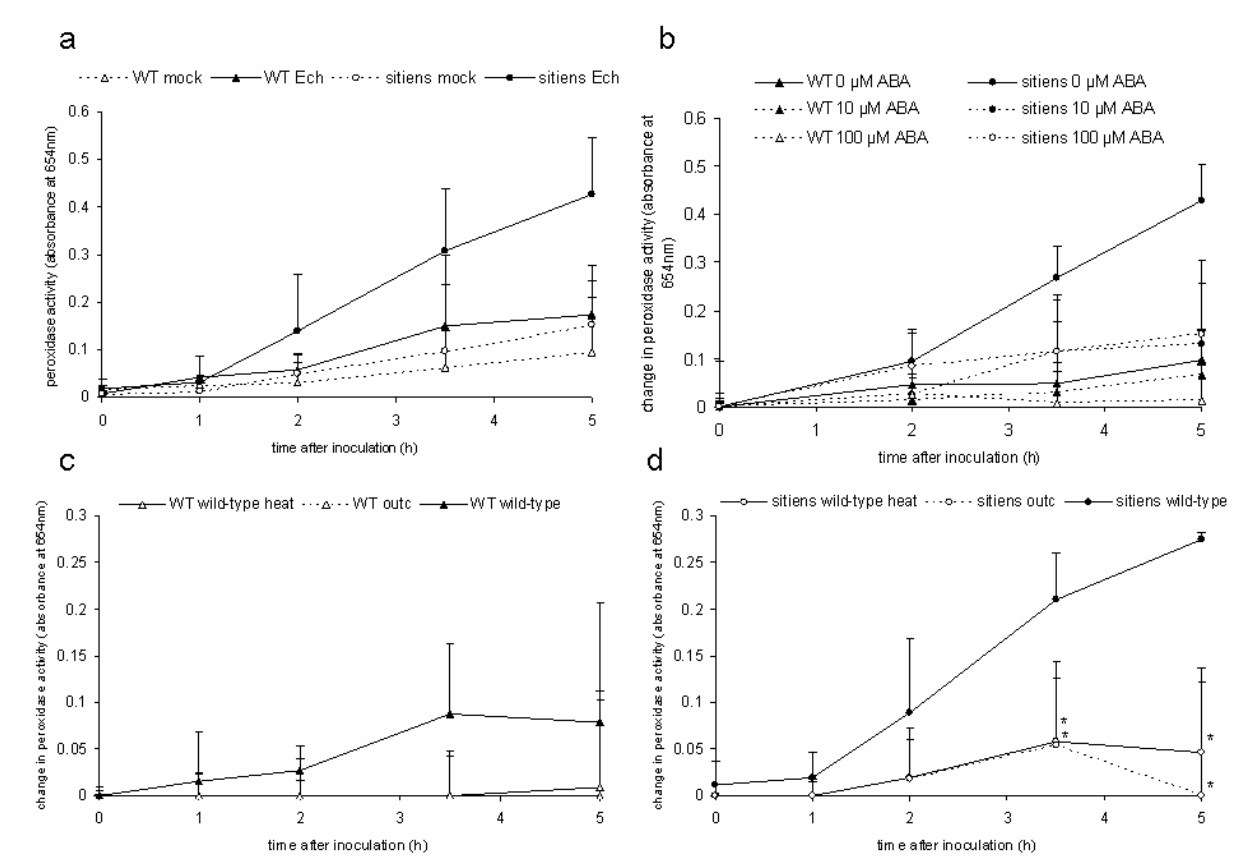

Figure V-8: Extracellular peroxidase activity in wild type (WT) and *sitiens* infiltrated with *E. chrysanthemi* pectinase-containing culture filtrate.
(a): Leaf discs were sampled from leaves infiltrated with the culture filtrate (CF) of *E. chrysanthemi* grown on a medium containing PGA to induce pectinase production ("*Ech*") or with pure medium ("mock"). (b): Leaf discs were sampled from wild type and *sitiens* infiltrated with CF four hours after a single spray with 0, 10 or 100 µM ABA. All spray solutions contained 0.05% ethanol. (c, d): Leaf discs were sampled from wild-type (c) and *sitiens* (d) leaves infiltrated with CF of *E. chrysanthemi* strains with functional type II secretion ("wild-type") and type II secretion deficiency ("outC"). In addition, heat-treated (80°C for 15 minutes) pectinase-containing CF ("wild-type heat") was used. In (b), (c) and (d), the presented change in peroxidase activity is the absolute activity value after subtraction of the activity level of medium-infiltrated samples for each time point. During sampling, discs were fixed in ethanol and peroxidase activity was measured at 654 nm after addition of TMB and 0.03% H_2O_2. The mean + standard error of the absorbance of the incubation solution from four discs of different plants are presented. In (c) and (d), significant differences at p=0.05 between "wild-type" and the two other treatments were determined by using one-way ANOVA and Duncan post-hoc tests for each time point and are indicated with "*". All experiments were repeated with similar results.

DISCUSSION

Tomato defence responses upon *E. chrysanthemi* infection are controlled by ABA

This work shows that infiltration of tomato leaves with *Erwinia chrysanthemi* resulted in both local and systemic infection of wild-type tomato plants, whereas there was little or no infection of ABA-deficient *sitiens* mutant plants. These results are in agreement with the earlier observations that *sitiens* is more resistant to *B. cinerea* (Audenaert et al., 2002a), *O. neolycopersici* (Achuo et al., 2006), and *P. syringae* pv. *tomato* (Thaler and Bostock, 2004). Furthermore, exogenous application of ABA suppressed the resistance to *E. chrysanthemi* in *sitiens* and significantly increased disease severity on wild type. While exogenous ABA also restored susceptibility in *sitiens* to *B. cinerea* and *O. neolycopersici* to wild-type levels, it did not further increase disease severity in wild-type tomato (Achuo et al., 2006).

The *sitiens* tomato mutant differs from the wild type in growth rate, plant development and water relations. The mutant exhibits a higher transpiration rate and a lower leaf water potential and turgor, resulting in reduced leaf expansion and a more compact leaf structure (Nagel et al., 1994; see supplementary figure V-S1). These morphological differences, however, do not account for the observed resistance in *sitiens*, since not only ABA supplementation during growth but also a pulse treatment with ABA prior to *E. chrysanthemi* infiltration completely restores wild-type susceptibility. In addition, *sitiens* plants grown at high temperature and RH (±100%) (conditions in which morphological differences are no longer visible) are still highly resistant to *E. chrysanthemi*.

The possibilities that *E. chrysanthemi* resistance in *sitiens* is due to inhibition of bacterial growth or to an inability of *E. chrysanthemi* CWDEs to degrade *sitiens* cell walls were also excluded. However, this work demonstrates that *E. chrysanthemi* leaf infiltration triggers an active defence response in *sitiens*, involving accumulation of extracellular hydrogen peroxide (H_2O_2) and a faster and stronger increase in peroxidase activity compared to wild-type tomato.

Pathogen containment is a key event in the resistance of *sitiens* to *E. chrysanthemi*

It has been shown before that H_2O_2 and peroxidase production are implicated in plant - *Erwinia* interactions. Wu et al. (1995) reported increased resistance to *E. carotovora* subsp. *carotovora* in transgenic potato plants expressing a H_2O_2-generating glucose oxidase, and also peroxidase activity is known to increase during *Erwinia* infection (Wegener, 2002), although transgenic over-expression of peroxidases does not always result in increased resistance to this pathogen (Ray et al., 1998). In addition, some sweetpotato peroxidase genes were expressed during *E. chrysanthemi* infection, while others were not, pointing to specificity of peroxidase isozymes in defence against *E. chrysanthemi* (Jang et al., 2004).

We did not find evidence for a direct antimicrobial role of H_2O_2, since *E. chrysanthemi* grew to a similar extent in wild type and *sitiens* prior to maceration. In addition, Miguel et al. (2000), who studied the mechanisms involved in the protection of *E. chrysanthemi* against oxidative stress, proved that there is no direct antimicrobial effect of host-produced H_2O_2 in potato and tobacco. Moreover, *E. chrysanthemi pecS* mutant, which is an overproducer of the antioxidant indigoidine and was shown to be more resistant to oxidative stress (Reverchon et al., 2002), was not able to cause sustained maceration symptoms on *sitiens*, which again opposes a direct effect of H_2O_2 on *E. chrysanthemi* survival *in planta*. Instead, extracellular H_2O_2 in *sitiens* specifically accumulated at the sites where bacterial progression was arrested, which points to a role of this accumulation in bacterial containment, because in wild-type tissue neither this reaction nor the halt of bacteria was observed.

During an oxidative burst, H_2O_2 is used as a substrate for oxidative polymerisation of various cell wall components controlled by the peroxidative cycle of extracellular peroxidases. By this mechanism, plant peroxidases are essential to restrict pathogen progress because of their cell wall cross-linking activity in the formation of lignin, extensin cross-links and dityrosine bonds (Passardi et al., 2005). In the present work, accumulation of stains for protein cross-linking and for incorporation of phenolic compounds in the cell wall was detected at the borders of *E. chrysanthemi* lesions in *sitiens*, again indicating that active containment of bacteria to the infiltrated zone might be an important mechanism for *sitiens* to limit *E. chrysanthemi*.

Source of *sitiens* H_2O_2 accumulation during *E. chrysanthemi* infection

Besides their prominent role in peroxidative cell wall modification, plant cell wall peroxidases can have a function in regulating H_2O_2 levels and releasing ROS through a hydroxylic cycle (Passardi et al., 2005). Moreover, extracellular peroxidases are considered to function together with membrane-bound NADPH oxidases as the two major mechanisms of ROS production during an oxidative burst (Bolwell and Wojstaszek, 1997). Time-course experiments in *sitiens* revealed that the increase in peroxidase activity (at 8 hpi) occurs earlier than the first detection of H_2O_2 accumulating (at 12 hpi). However, our data do not allow appointing peroxidases as the main H_2O_2-source in *sitiens* during infection given that the TMB-peroxidase assay is much more sensitive than the DAB staining technique (Rye et al., 1984), which can explain the temporal shift in detection. On the other hand, in a recent model, Bindschedler et al. (2006) propose that apoplastic peroxidases are a general initial rapid source of ROS, and are essential for conferring at least partial resistance independently of any involvement of NADPH oxidases in a later stage. Other cellular compartments have been proposed for ROS generation during plant-pathogen interactions (Bolwell and Wojstaszek, 1997). Treatment of potato with the CWDE-containing CF of *E. carotovora* resulted in down-regulation of photosystem I and H_2O_2 accumulation in chloroplasts (Montesano et al., 2004). In addition to the extracellular H_2O_2 that was specifically present in *sitiens* lesion borders, H_2O_2 was also detected in chloroplasts within the infiltrated zone of both wild-type and *sitiens* tomato leaf tissue. Based on this work and others however, we found no indications that H_2O_2 accumulation in chloroplasts is more than just a consequence of *Erwinia* symptom development, and it is doubtful if this reaction significantly contributes to enhanced defence.

Sitiens defence activation is triggered by type II-secreted proteins

In most plant pathogenic bacteria, plant defence responses were mainly reported to be elicited by effector proteins injected in plant cells by the type III secretion system. Although *E. chrysanthemi* possesses a hypersensitive response and pathogenicity (Hrp) type III secretion system (Bauer et al., 1994), it was shown in the present work that defence responses in *sitiens* plants are activated by type II secreted proteins. Different proteins might be the elicitors of these plant responses. First, analysis of the *E. chrysanthemi* secretome revealed that an Avr-like protein is secreted by the Out system (Kazemi-Pour et al., 2004). In addition to this protein, other type II-secreted proteins produced during culture under conditions that induce pectinase

production, as used in this work, are essentially pectinases (Kazemi-Pour et al., 2004). These enzymes could interact with plants in two ways: either they could be directly recognized as elicitors, as it has been shown for the PelI protein that can cause a response on tobacco independently of its pectinolytic activity (Shevchik et al., 1998), or they could produce elicitors indirectly by enzymatic degradation of the plant pectin matrix. Since heat-degradation resulted in loss of the eliciting activity, the conformational integrity of the protein appears to be important for elicitation. Because protein elicitors like harpins are heat-stable for their activity (reviewed in Cornelis and Van Gijsegem, 2000), this points more to the involvement of pectinolytic activity to produce cell wall degradation products eliciting *sitiens* peroxidase activation. Indeed, recognition of oligogalacturonides (OGAs) by plants triggers defence responses within minutes, including an oxidative burst and cell wall strengthening by peroxidases (Ridley et al., 2001), and plant cell wall degradation by *E. chrysanthemi* pectinases results in the release of pectic oligomers of different size dependent of the acting enzyme (Roy et al., 1999).

Two hypotheses may explain the influence of ABA on *sitiens* defence responses. Firstly, basal ABA levels in wild-type tomato plants may suppress the fast and more powerful reaction to the OGA signal or other putative Out-related signals observed in *sitiens*. This view is supported by the limited increase in peroxidase activity in *sitiens* plants that were pulse-treated with ABA before pathogen inoculation. However, ABA pulse treatment did not alter the initial *sitiens* peroxidase activation at 8 hpi, indicating that morphological changes resulting from ABA-deficiency during growth can also have a significant effect on elicitation of defence responses. This is consistent with the stronger increase of disease after ABA supplementary feeding than after a single ABA spray. Therefore, as a second possibility, it can be postulated that CWDEs may release different types of OGAs in *sitiens* and wild type. ABA is known to influence cell wall composition in various ways, including alteration of the pectin matrix (Micheli et al, 2001; Lohani et al., 2004). The biological activity of OGAs strongly depends on the degree of polymerisation (PD) and esterification (Ridley et al., 2001). Specifically for *E. chrysanthemi*, it was suggested that the release of OGAs with a low PD stimulates induction of pectinase production, while OGAs with a higher PD (10 to 15 residues) activate plant defence systems, which indicates that the outcome of an interaction, i.e. resistance or susceptibility, might be influenced by the type of oligomers surrounding the bacterium (Barras et al., 1994). An alternative link between ABA and cell wall composition was recently provided by Hernández-Blanco et al. (2007), who found an enhancement of ABA-responsive defence-related gene activation in Arabidopsis plants mutated in cellulose synthase genes required for

secondary cell wall formation. We are currently studying the effect of ABA on cell wall modifications by comparing the cell wall composition of wild-type and *sitiens* tomato leaves (see chapter 6).

MATERIALS AND METHODS

Plant material and growing conditions

Seeds of tomato cv. Moneymaker and the ABA-deficient *sitiens* mutant (Taylor et al., 1988), provided by Prof. M. Koornneef (Wageningen University, The Netherlands), and were sown directly in pots containing 150 g general-purpose potting soil and seedlings were thinned to one plant per pot after sprouting. Two different experimental growing conditions were used: (1): growth chamber (day/night temperature of 28±1°C/26±1°C; 16H light; RH of ±100%) and (2) greenhouse (day/night temperature of 21±1°C/18±1°C; 16H light; RH of ±65%).

Bacterial inoculum and plant inoculation

Three different *Erwinia chrysanthemi* Burkholder *et al.* strains (now reclassified in *Dickeya* species by Samson et al. (2005)) derived from wild-type strain 3937 were used in this study: the *gfp9* strain that was selected as a constitutive producer of the GFP protein by genomic insertion of the *gfp* mini-transposon pAG408 (Suarez et al., 1997), a *pecS* strain carrying a mutation in the global transcriptional regulator *pecS* gene, that leads to derepressed synthesis of several pathogenicity factors (Reverchon et al., 1994) and an *outC* mutant strain, deficient in the type II secretion system, constructed by transduction of the *outC*::*uidA*-Km fusion (Kazemi-Pour et al., 2004) into the 3937 wild-type strain.

For all bacterial plant infiltrations, *E. chrysanthemi* was grown overnight on Luria-Bertani (LB) medium (Bertani, 1951) and bacterial cells were collected from the agar plates by washing with 50 mM KCl. Bacterial concentration was determined with a spectrophotometer at 600 nm and the inoculum solution prepared by dilution to 10^6 CFU/ml or 10^7 CFU/ml in 50 mM KCl. The second-youngest leaves of plants at 3-4 leaf stage were inoculated by syringe infiltration on intact plants. Two to three leaflets of the same leaf were infiltrated per plant, using at least six plants per treatment. Control plants were inoculated with 50 mM KCl. Leaf infiltration was done by gently pushing the bacterial cell suspension onto the adaxial surface of

the leaf using a 1ml-syringe without needle. Infiltrated plants were incubated in humid boxes at 28°C.

Disease evaluation

A disease evaluation scheme was developed for *E. chrysanthemi* inoculation on tomato, based on the nature of symptoms produced locally on the inoculated leaf tissue and those produced on plant parts distant from the point of inoculation. Local disease symptoms were evaluated on the inoculated leaves at 24, 48 and 72 hours post inoculation (hpi) on a 0 – 3 scale: (0): no disease symptoms; (1): necrosis of the infiltrated tissue (Fig. V-1b); (2): maceration contained within the infiltrated tissue (Fig. V-1a); (3): spreading maceration beyond the infiltrated tissue (Fig. V-1c and 1d). Data were analyzed by the Kruskal-Wallis/Mann-Whitney nonparametric tests in SPSS.

At 7 days post inoculation plants were evaluated for appearance of symptoms on plant parts distant from the infiltrated leaf (systemic infection) (Fig. V-1f and 1g).

Exogenous abscisic acid treatment

Two methods of exogenous abscisic acid (ABA) treatment were applied: (i): physiological supplementation whereby two-week-old plants were sprayed with 100 μM ABA at five days intervals for 25 days (six treatments in all) and (ii): pulse application by spraying plants with 10 or 100 μM ABA, 4 hours before bacterial inoculation. ABA solution was prepared from a stock of 10 mM in 5% ethanol and the final ethanol concentration in the treatment solution was 0.05%. Control plants were treated with water containing 0.05% ethanol. Plants were treated by foliar spray with a fine mist of the solutions to near run-off. The physiological supplementation treatment was intended to restore the morphology of *sitiens* plants to the wild-type phenotype, while the pulse application was intended to raise ABA levels in the plants without any morphological changes.

Quantification of bacteria in plant tissue

To compare the survival and multiplication of the bacteria in infiltrated tomato leaf tissue, 5 mm diameter leaf discs were cut out from the infiltrated zone using a cork borer. The

samples were ground individually in 50 mM KCl and plated out on LB medium in a 10-fold dilution series with the drop-plate method (Herigstad et al., 2001). For each dilution in the series, three 10 µl-droplets were placed on LB plates and incubated at 28 °C for 24 hours. *E. chrysanthemi* was identified on the basis of colony morphology and the presence of strain *gfp9* was verified based on green fluorescence under UV-light. Colony-forming units (CFU) were counted on plated droplets that contained between 7 and 70 colonies and the number of CFU per mm^2 of leaf area was calculated and compared statistically by the t-test for pairwise comparisons of means. In the experiments in which wild-type *E. chrysanthemi* strain growth was compared for wild type and *sitiens*, at least five leaf discs from different plants of each genotype were sampled at 0, 4, 8, 12, 24 and 48 hpi. At time points later than 12 hpi, samples were taken separately for macerated and non-macerated leaf tissue. In the experiment in which growth of different *E. chrysanthemi* strains was compared, three leaf discs from different plants of each genotype were sampled at 0, 8 and 12 hpi.

Preparation and application of *E. chrysanthemi* cell wall degrading enzymes-containing culture filtrate

E. chrysanthemi was grown at 28°C on liquid pectinase-inducing medium (30 mM K_2PO_4, 8.3 mM NaH_2PO_4, 1.6 mM $MgSO_4$, 50 mM $(NH_4)_2SO_4$, 0.5% casamino acids (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 0.5% polygalacturonic acid). At the end of the exponential growth phase the cultures were centrifuged at 7000 × *g* for 10 minutes. The supernatant containing the cell wall degrading enzymes (CWDEs) was filter-sterilised using a Millex®GV 0.22 µm filter unit (Millipore Corporation, Bedford, MA, USA) and a five fold dilution series in the same medium without PGA was prepared.

Two different set-ups were used to infiltrate the culture filtrate (CF) containing the CWDEs with a syringe into the second-youngest leaves of tomato plants at the 3-4 leaf stage. The first consisted in using intact plants, which were incubated in humid boxes at 28°C, as in plant infection trials. In a second set-up, the CF was infiltrated into detached leaflets that were incubated on a moistened filter paper in sealed Petri dishes at 37°C, using one leaflet per plant.

To compare cell wall degradation in *sitiens* and wild type, the infiltration zones were evaluated for appearance of maceration at 2-to-4 hour intervals during 24 hours. For each CF concentration, the presence or absence of cell wall degradation was scored on six leaflets of each genotype at the various time points. From this data, the time required for half of the

leaflets to show macroscopically visible cell wall degradation was extrapolated and plotted against the CF dilution factor.

Staining and microscopy procedures

For H_2O_2 accumulation, staining was performed according to the protocol of Thordal-Christensen et al. (1997). Three hours before each sampling time point, leaf discs that contained part of the *E. chrysanthemi* infiltration zone were cut out of the leaf with a 1-cm diameter cork bore and placed in standard 24-well plates. Immediately, the discs were floated in a solution of 1 mg/ml 3,3'-diaminobenzidine (DAB)-HCl (pH 4) (Sigma). Polymerisation of the DAB molecule at the site of H_2O_2 and peroxidase accumulation displays a brown reddish colour that is macroscopically visible and, because of the high spatial and temporal distribution of the oxidised DAB molecule, it can be visualised under bright-field microscopy. After staining, the discs were fixed and cleared in 100% ethanol and mounted on microscopy slides for evaluation.

Representative parts of DAB-stained *E. chrysanthemi* lesion borders were embedded in Technovit 7100 histo-embedding medium (Heraeus Kulzer, Wehrheim, Germany) according to the manufacturer's descriptions and semi-thin sections (4 µm) were cut with a Leica RM2265 motorised rotary microtome (Leica Microsystems, Nussloch, Germany). A subset of the sections was stained with 1% toluidine blue for 1 minute to visualise bacteria, chloroplasts and plant cell walls, while the remaining sections were evaluated without additional staining, allowing for ultra-structural detection of H_2O_2.

For protein cross-linking, staining was performed as described by Mellersh et al. (2002). Ethanol-fixed leaf discs containing part of the infiltrated zone were placed in 1% SDS at 80°C for 24 h and stained with 0.1% Coomassie blue in 40% ethanol/10% acetic acid for 15 min and subsequently washed in 40% ethanol/10% acetic acid. Cell wall fortification was visualised after safranin-o staining according to Lucena et al. (2003). Leaf discs were incubated in 0.01% safranin-o in 50% ethanol for 3 min.

Measurement of peroxidase activity

Extracellular peroxidase activity was measured with the TMB assay based on Ros Barceló (1998). One-cm leaf discs containing part of the *E. chrysanthemi* infiltration zone were cut from the leaf as described above and fixed in pure ethanol. After subsequent washing in

distilled water, the discs were incubated in 1.5 ml of a 50 mM tris-acetate buffer (pH 5.0) containing 0.1 mg/ml 3,5,3',5'-tetramethylbenzidine (TMB) (Sigma) and 0.03% H_2O_2 for 20 minutes. Peroxidase activity of the discs was determined by measuring the absorbance of the incubation solution at 654 nm and compared statistically by one-way ANOVA and Duncan post-hoc tests.

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SUPPLEMENTARY MATERIAL

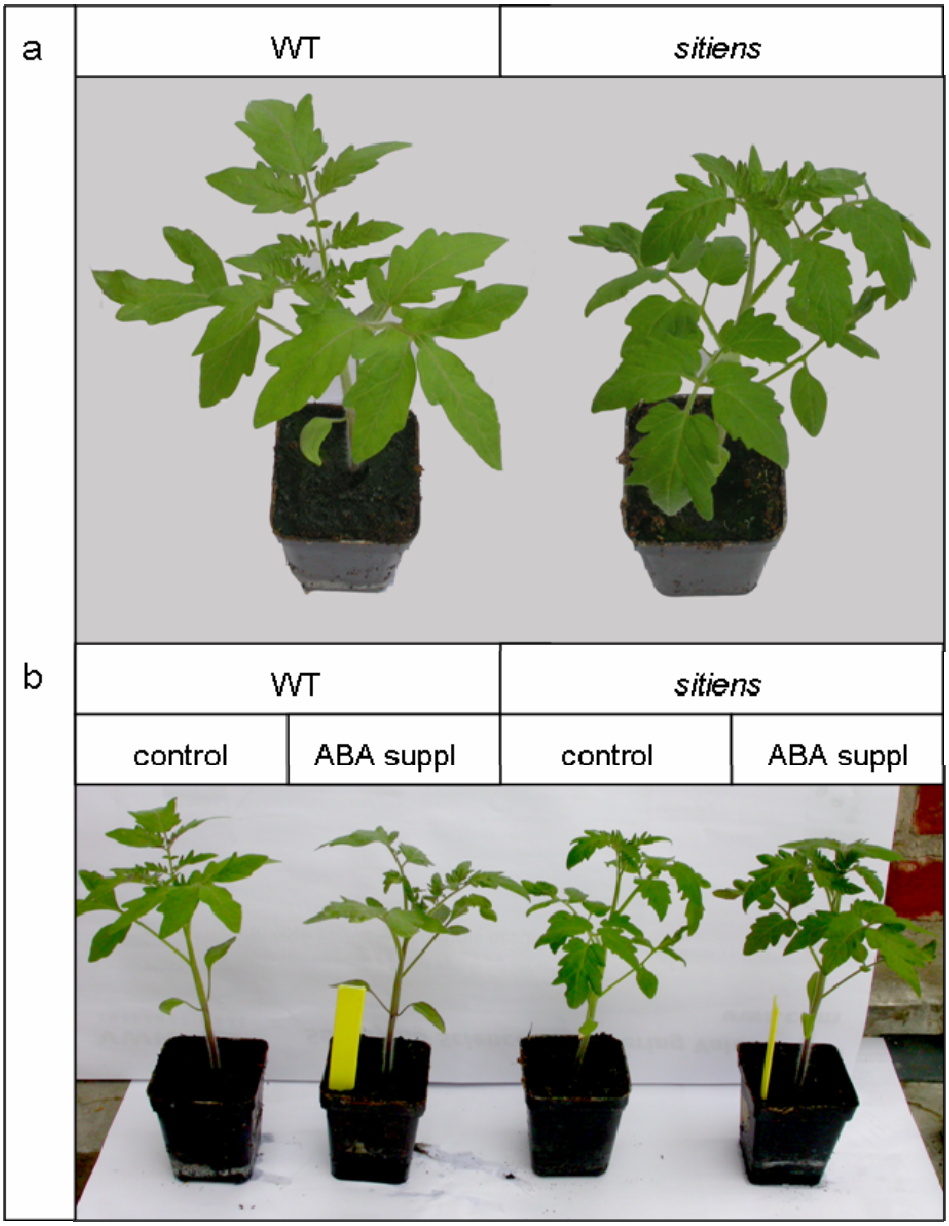

Figure V-S1: ABA-related differences in plant morphology on tomato grown under greenhouse conditions. Plants were grown at day/night temperature of 21±1°C/18±1°C, 16H light and RH of ±65% for 5 weeks. (a): Comparison of wild-type (WT) and *sitiens*. ABA deficiency in *sitiens* results in reduced leaf expansion and in a higher number of leaves. The background of the image was modified with Adobe Photoshop Elements software. (b): Effect of repeated exogenous ABA spraying (100 µM) during plant development of *sitiens* and wild-type (WT) tomato ("ABA-suppl" treatment). ABA supplementation of *sitiens* restored wild-type plant morphology and ABA supplementation of wild-type plants further increased leaf expansion.

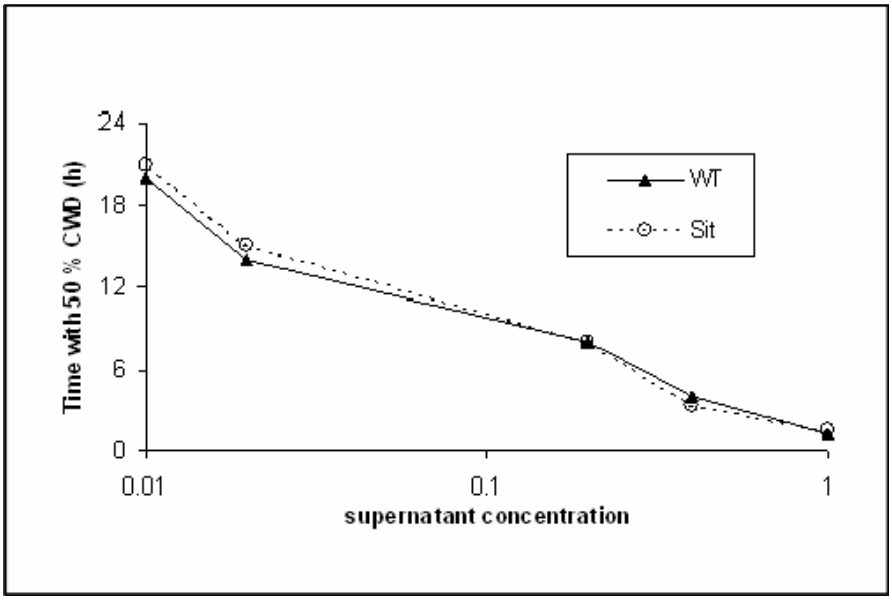

Figure V-S2: Cell wall degradation in *sitiens* and wild-type (WT) tomato tissue after application of *E. chrysanthemi* pectinases.
Pectinase production was induced on a medium containing PGA and different concentrations of the bacteria-free culture supernatant were infiltrated into detached leaves and incubated at 37°C. Six leaflets were used for each supernatant concentration and presence or absence of cell wall degradation was scored for each leaflet at 2–to-4-hour-intervals during 24 hours. The time point at which 50% of the leaflets were showing cell wall degradation was extrapolated and plotted for each genotype.

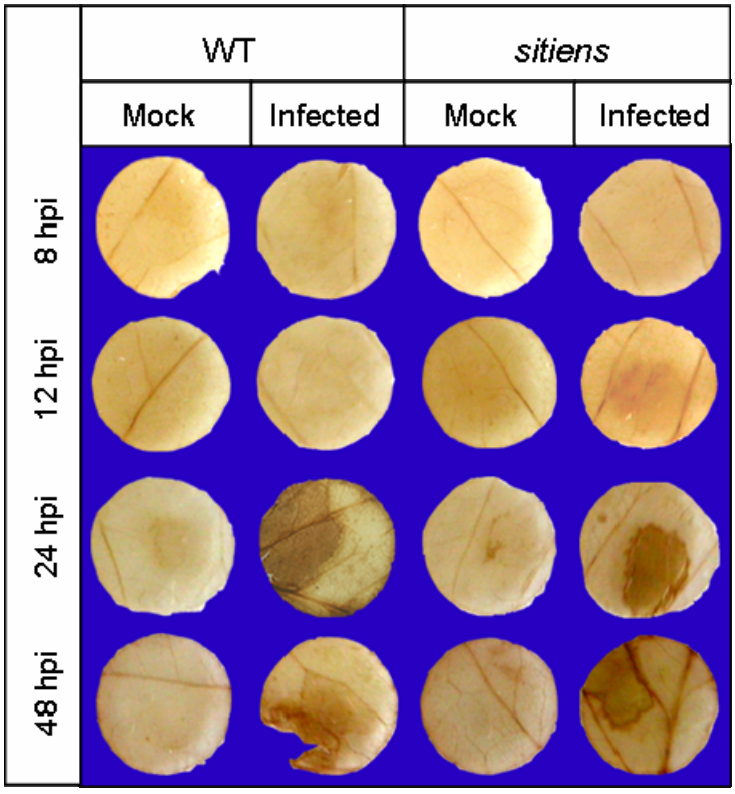

Figure V-S3: Temporal evolution of H_2O_2 accumulation in wild-type (WT) and *sitiens* tomato after infection with *E. chrysanthemi*.
DAB staining of leaf discs infiltrated with 10^6 CFU ml^{-1} ("infected") or 50mM KCl ("mock") was performed at different time points postinoculation (8, 12, 24, and 48 hpi). One representative disc out of three replicates is shown for each time point. The experiment was repeated with similar results.

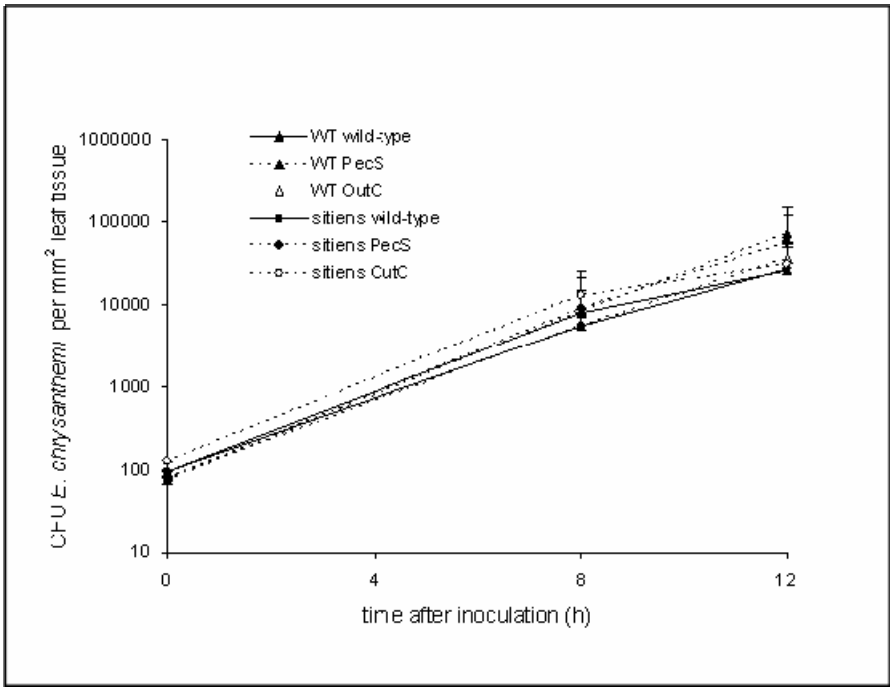

Figure V-S4: Survival and growth of *E. chrysanthemi* strains with different pectinase production in wild-type (WT) and *sitiens* leaf tissue.
Leaf discs were sampled at different time points from leaves infiltrated with 10^6 CFU ml^{-1} of *E. chrysanthemi* wild - type strain, the pathogenicity factor overproducer *pecS* strain and the type II secretion-deficient *outC* strain. After crushing in 50 mM KCl and plating out on LB medium, samples were incubated at 28 °C for 24 hours and counted. Data presented are the means + the standard error using 3 plants per treatment. Bacterial counts at each time point were compared statistically by the t-test and no significant differences were found.

Chapter

6

Immunolocalisation of Pectin, Extensin and Arabinogalactan Proteins in *sitiens* and Wild-Type Tomato

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We have previously demonstrated that the ABA-deficient *sitiens* tomato (*Solanum lycopersicum*) mutant is highly resistant to the necrotrophic pathogens *B. cinerea* and *E. chrysanthemi* (see chapters 2 and 5). It was speculated that ABA deficiency in *sitiens* results in differential recognition and/or early signalling events within the cell wall, leading to an efficient cell wall fortification response (see chapters 3 and 5). In the present chapter, we aim to expand our knowledge on the cell wall-related features that allow *sitiens*' hyperinduction of defence by evaluating the presence and abundance of several cell wall components in an immunolocalisation study.

We have compared pectin content and composition on a histological and ultrastructural level between *sitiens* and wild-type by using JIM5 and JIM7 monoclonal antibodies that bind specifically to different pectin epitopes. Whereas wild-type tomato contains high concentrations of homogalacturonan in outer periclinal cell walls, the *sitiens* mutant has a more uniform distribution of homogalacturonan over all types of cell walls. We have also assayed the presence of extensins (using the LM1 antibody), which are important for the formation rigid impermeable protein networks during cell wall fortification, but found no differences between wild type and *sitiens*. Furthermore, we have evaluated the distribution of arabinogalactan proteins by the use of antibody LM2. Arabinogalactan proteins are prime candidates for expediting cell wall-related pathogen defence by mediating signalling at the cell surface. LM2 binding was strongly present in chloroplastic membranes of wild type, while in *sitiens*, binding of LM2 to chloroplastic membranes was much less pronounced. Instead, the LM2 epitope in *sitiens* was found in intracellular aggregates, suggesting LM2 binding to intracellular vesicles. The possible consequences of these differences between *sitiens* and wild-type in relation to pathogen recognition and early defence signalling are discussed.

INTRODUCTION

Cell walls are vital for a plant's construction by providing cells with mechanical strength and by keeping cells in shape. The major chemical components of cell walls are cellulose, hemicellulose, pectic compounds, proteins, glycoproteins and lignin. Molecules of these cell wall components are linked with hydrogen bonds, ionic bonds, hydrophobic interactions and covalent bonds, together forming intricate interconnected networks. The pectic compound network constitutes of complex and heterogeneous polysaccharides that contain 1,4-linked α-D-galactosyluronic acid residues, including homogalacturonan, rhamnogalacturonan-I and substituted galacturonans (rhamnogalacturonan-II). Pectins form about 35% of the dry weight of dicot cell walls and the pectin network provides the cell wall with a certain degree of plasticity. Some of the carboxyl groups of the linear galacturonan backbones are methylated or contain side chains. The localisation and structure of pectin has an impact on plant cell growth and development and response to external stimuli (Perez et al., 2000; Ridley et al., 2001).

Plant cell walls have a wide range of important functions in encountering plant pathogens. Most simply, cell walls provide a physical barrier separating pathogens from the internal content of plant cells. Cell walls are also dynamic reservoirs of antimicrobial proteins and secondary metabolites that inhibit the growth of many pathogens (Vorwerk et al., 2004). In addition, pathogen recognition leads to induction of site-specific cell wall fortification, consisting of rapid cross-linking of preformed cell wall molecules and *de novo* synthesis and deposition of secondary wall components (Bradley et al., 1992; see also chapter 2). Oxidative cross-linking of extensins, which are hydroxyproline-rich cell wall glycoproteins, results in a dense extensin network that has a key function in increasing cell wall rigidity and resistance to pathogen lytic enzymes (Ribeiro et al., 2006). Furthermore, a surveillance system for cell wall integrity appears to sense perturbation of the cell wall structure upon pathogen attack and is interconnected with known plant defence signalling pathways. Plant cell wall degradation by pathogens leads to the release of cell wall breakdown products, which are recognised by the plant as endogenous elicitors that form signals to activate defences (Schulze-Lefert, 2004). Molecular interactions and cellular signalling at the cell surface presumably involve arabinogalactan proteins (AGPs). AGPs are a family of extensively glycosylated hydroxyproline-rich glycoproteins, many of which contain glycosylphosphatidylinositol (GPI) lipid anchors (Showalter, 2001; Knox, 2006; Seifert and Roberts, 2007). It was speculated that GPI-anchored AGPs provide a link between pathogen-induced changes in extracellular matrix

polysaccharides and cell signalling (Seifert and Roberts, 2007).

We have shown previously that ABA-deficient *sitiens* tomato (*Solanum lycopersicum*) mutants are highly resistant to the necrotrophic pathogens *B. cinerea* and *E. chrysanthemi* (see chapters 2 and 5). *Sitiens* resistance to both pathogens was shown to result from fast and strong activation of hydrogen peroxide-dependent cell wall fortification. It was speculated that ABA deficiency in *sitiens* results in differential recognition and/or early signalling events within the cell wall, leading to an efficient cell wall fortification response (see chapters 3 and 5).

Both *E. chrysanthemi* and *B. cinerea* have developed a sophisticated enzymatic machinery to break down cell walls, and require especially the enzymes for pectin decomposition as pathogenicity factors (Alghisi and Favaron, 1995; Van Kan 2006). *B. cinerea* has at least six endopolygalacturonase genes, of which some are required for full virulence (ten Have et al., 1998; ten Have et al., 2001). Furthermore, *B. cinerea* possesses pectin methylesterases to facilitate the action of polygalacturonases on highly methylated pectin (Kars et al., 2005b; Van Kan, 2006). Also the pectinase equipment of *E. chrysanthemi* is particularly well characterised. *E. chrysanthemi* strain 3937 produces at least six endo-pectate lyases, an exo-pectate lyase, two pectin methylesterases, a pectin lyase, and an exopolygalacturonase (Collmer and Keen, 1986; Kotoujansky, 1987; Barras et al., 1994; Hugouvieux-Cotte-Pattat, 1996).

Experiments with *E. chrysanthemi* pectinase-containing culture filtrate have shown that *sitiens* cell walls are degradable by *E. chrysanthemi* pectinases and that no clear qualitative or quantitative differences between wild-type and *sitiens* tomato were found in cell wall degradability (see chapter 5 figure V-S2). On the other hand, it was demonstrated in chapter 5 that *sitiens* defences are only strongly activated in the presence of non-denaturated *E. chrysanthemi* type II secreted proteins, which consist mainly of pectinases. Considering that pectin oligogalacturonide (OGA) breakdown products are strong elicitors of pathogen defence responses, it was hypothesised that alterations in *sitiens* cell wall composition, and more specifically in pectic components, could result in stronger and/or faster elicitation of defence. Preliminary measurements of *sitiens* and wild-type cell wall composition by use of gas-chromatography coupled mass spectrometry revealed that *sitiens* cell walls of tomato leaves contained less galactose (in homo- and rhamnogalacturonan of pectic substances), compensated by an increase in other monosaccharides, but that the galactose fraction in *sitiens* was slightly more methylated compared to the galactose in wild-type pectic substances (Katrien Curvers, personal communication; Stage report Katrien Curvers INRA – Versailles November 2006-

januari 2007). Furthermore, Fourier-transform infrared micro-spectroscopy (Mouille et al., 2003) was performed on different types of *sitiens* and wild-type samples (stem sections, xylem cells, lower and upper leaf epidermis) to identify differences in cell wall composition, but no conclusive statements could be made due to high variability in the results (Katrien Curvers, personal communication; Stage report Katrien Curvers INRA – Versailles November 2006-januari 2007). Immunolocalisation with antibodies that recognise specific structural features of primary cell wall pectin are powerful tools for determining the localisation and distribution of these polysaccharides (Ridley et al., 2001).

In this chapter, we aim to compare *sitiens* and wild-type pectin content and composition on a histological and ultrastructural level by using monoclonal antibodies that bind specifically to different pectin epitopes. Furthermore, using immunological microscopy we evaluated the distribution of AGPs and extensins in *sitiens* and wild-type, because these two classes of glycoproteins are prime candidates for mediating enhanced cell wall-related pathogen defence by respectively mediating signalling at the cell surface or by forming rigid impermeable protein networks.

RESULTS

Pectin distribution in *sitiens* and wild-type leaves

Excised pieces of wild-type and *sitiens* tertiary leaves were embedded in LR white resin and semi-thin cross-sections were incubated with different pectin-binding monoclonal antibodies. Visualisation was done by antibody labelling with fluorescein isothiocyanate (FITC)-containing secondary antibodies. Monoclonal antibody JIM7 specifically recognises partially methylesterified epitopes of homogalacturonan, but does not bind to un-esterified homogalacturonan (Knox et al., 1990; Willats et al., 2000; Clausen et al., 2003). As expected, JIM7 bound to entire wall regions of epidermal, mesophyll and vascular cell types in both *sitiens* and wild type (Fig. VI-1). Immunological binding of the antibodies on cross-sections did not allow an absolute quantification of pectin content. Nevertheless, some surprising differences in JIM 7 binding intensity were consistent and clear between wild type and *sitiens*. In wild type, JIM7 binds especially strong to the outer periclinal walls of epidermal cells, i.e. cell walls in contact with the external environment, whereas binding intensity was much lower in walls of other wild-type cell types and in anticlinal and inner periclinal epidermal walls, i.e. cell walls

facing internal parts of the leaf (Fig. VI-1). In *sitiens*, differences in JIM7 antibody binding intensity between different cell types or cell wall regions were much less pronounced, resulting in a relatively uniform distribution of JIM7 on all leaf cell walls. These differences between wild-type and *sitiens* in relative binding intensity of the different wall regions were obvious in all samples that were examined. These results indicate that wild-type tomato contains high concentrations of partially methylated homogalacturonan in outer periclinal cell walls, whereas the *sitiens* mutant has a more uniform distribution of partially methylated homogalacturonan over all types of cell walls.

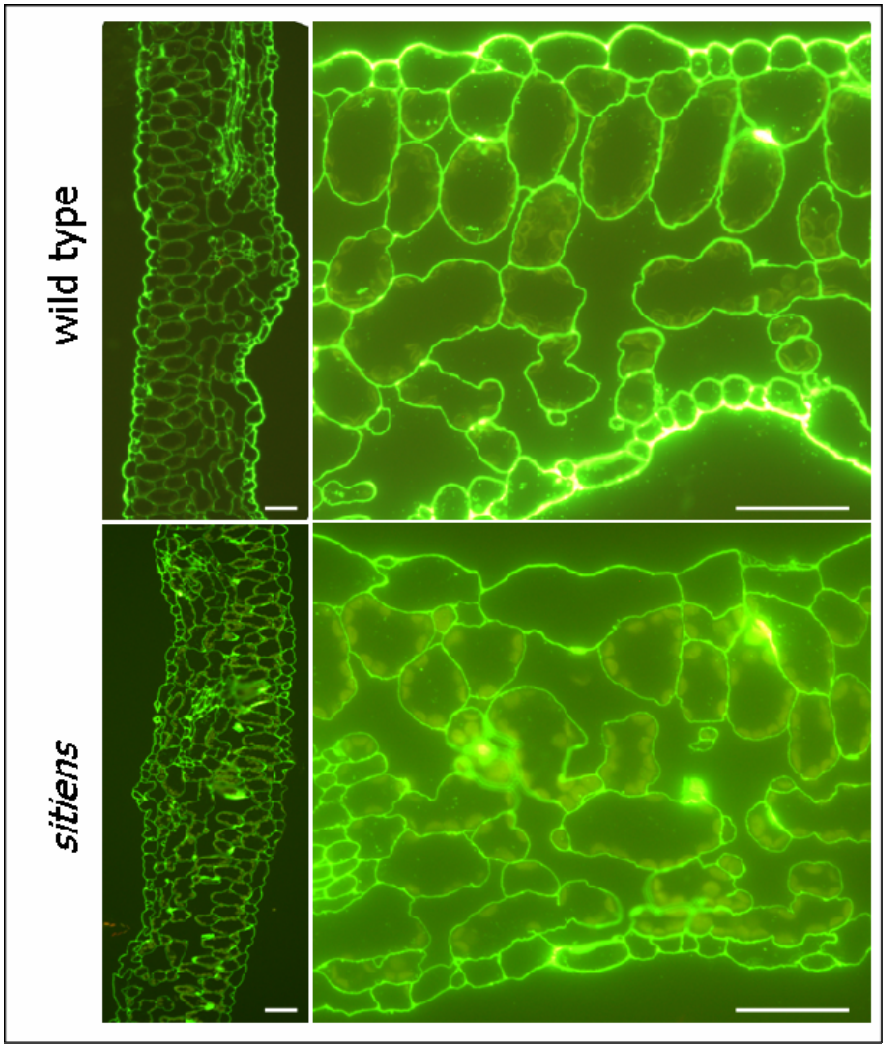

Figure VI-1: Immunolocalisation of partially methylesterified pectin with JIM7 monoclonal antibody in *sitiens* and wild-type tomato leaves. Semi-thin cross sections of excised parts of *sitiens* and wild-type leaves that were embedded in LR white resin were incubated with JIM7. Visualisation was done with epi-fluorescence microscopy after secondary labelling with FITC. At least 15 samples originating from different plants were examined for wild type and *sitiens*. Representative pictures of *sitiens* and wild type are shown. Scale bar = 50 µM.

Incubation of wild-type and *sitiens* samples was also performed with monoclonal antibody JIM5, which recognises partially methylesterified epitopes of homogalacturonan, and can also bind to un-esterified homogalacturonan (Knox et al., 1990; Willats et al., 2000; Clausen et al., 2003). JIM5 bound to cell wall regions of wild type and *sitiens* (Fig. VI-2). The differences in relative antibody binding intensity on different cell types or cell wall regions between *sitiens* and wild type, which were found for JIM7, were even more pronounced for

JIM5. Wild type displayed a much more intense signal in the walls of outer periclinal epidermal cells compared to other cell wall regions, whereas *sitiens* had a relative uniform distribution of JIM5 binding on all cell walls (Fig. VI-2). Furthermore, although absolute quantification of polysaccharide content is not possible by using this method, it seemed that in general JIM5 immunostaining was stronger in *sitiens* that in wild type, especially in wall regions other than those of outer periclinal epidermal cell walls (Fig. VI-2). These results were consistent on all samples that were examined.

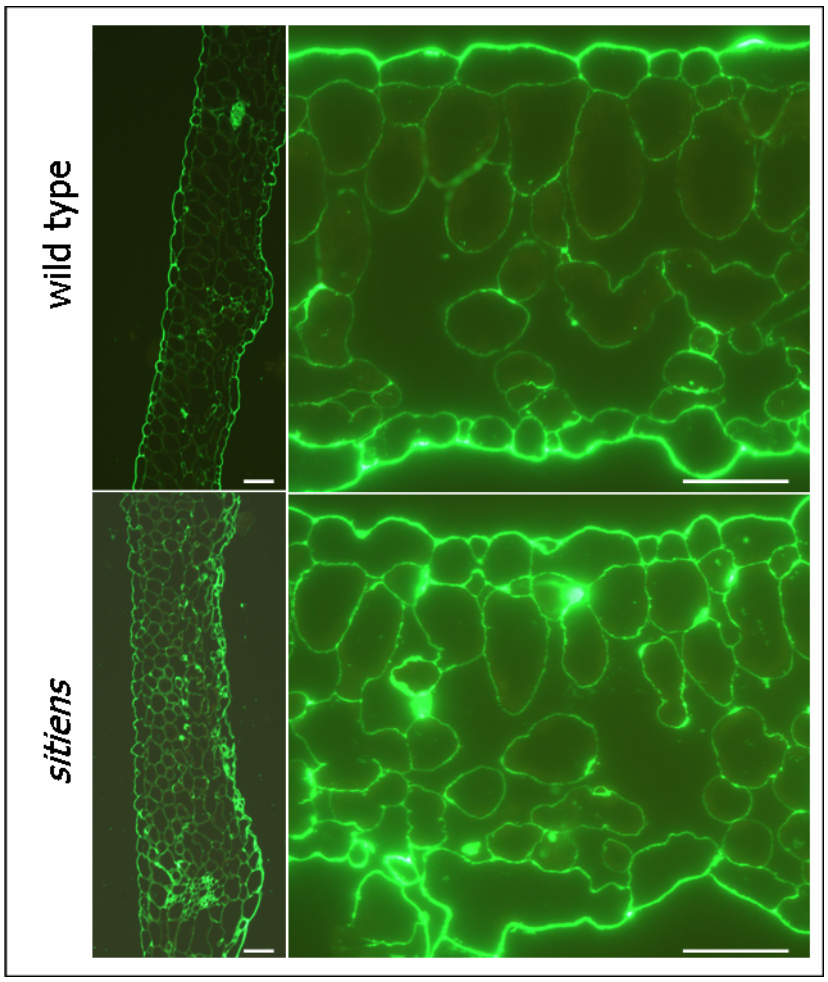

Figure VI-2: Immunolocalisation of partially methylesterified and un-esterified pectin with JIM5 monoclonal antibody in *sitiens* and wild-type tomato leaves. Semi-thin cross sections of excised parts of *sitiens* and wild-type leaves that were embedded in LRW resin were incubated with JIM5. Visualisation was done with epi-fluorescence microscopy after secondary labelling with FITC. At least 10 samples originating from different plants were examined for wild type and *sitiens*. Representative pictures of *sitiens* and wild type are shown. Scale bar = 50 µM.

Arabinogalactan protein distribution in wild-type and *sitiens* leaf tissue

To compare the localisation and content of arabinogalactan proteins in wild-type and *sitiens* leaves, we used the monoclonal antibody LM2 that recognises arabinogalactan proteins containing glucuronic acid residues (Smallwood et al., 1996; Yates et al., 1996). In both wild-type and *sitiens* leaf tissue the LM2 epitope was localised in plasma membrane regions (Fig. VI-3). In addition, LM2 strongly bound to intracellular compartments in wild-type and *sitiens*. Consistent differences between wild type and *sitiens* were found in the ultrastructural location of the LM2 epitope. In wild-type, LM2 binding was strongly present in chloroplastic membranes (Fig. VI-3). In *sitiens* however, binding of LM2 to chloroplastic membranes was much less pronounced. In stead, the LM2 epitope in *sitiens* was found in intracellular aggregates, suggesting LM2 binding to intracellular vesicles (Fig. VI-3).

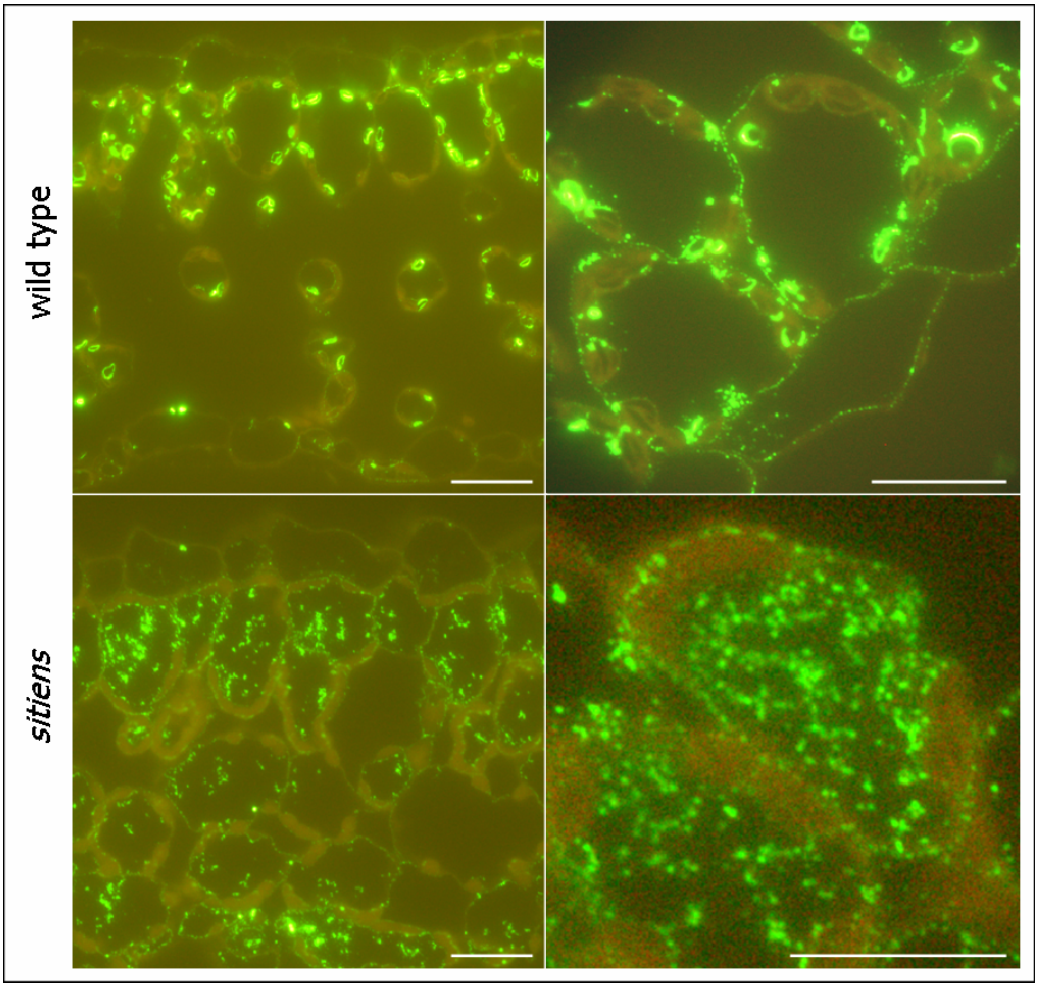

Figure VI-3: Immunolocalisation of arabinogalactan proteins with LM2 monoclonal antibody in *sitiens* and wild-type tomato leaves.
Semi-thin cross-sections of excised parts of *sitiens* and wild-type leaves that were embedded in LR white resin were incubated with LM2. Visualisation was done with epi-fluorescence microscopy after secondary labelling with FITC. At least 15 samples originating from different plants were examined for wild type and *sitiens*. Representative pictures of *sitiens* and wild type are shown. Scale bar = 50 µm.

To investigate the involvement of arabinogalactan proteins in ABA-regulated tomato defence reactions, we performed LM2 immunolocalisation after inoculation of *sitiens* and wild-type leaf discs with *B. cinerea*. Preliminary analysis of a low number of samples taken at 8, 16, 24 and 32 h after *B. cinerea* inoculation did not reveal clear differences for the different time points in the observations that were made. We chose 16 h after *B. cinerea* inoculation to evaluate more samples. In wild type, LM2 binding intensity either stayed unchanged after *B. cinerea* challenge (Fig. VI-4A), or decreased in tissue regions infected with *B. cinerea* (Fig. VI-4B and 4C). The decrease in LM2 binding could be observed as an abolishment of LM2 binding to chloroplastic membranes, whereas LM2 binding to the plasma membrane epitope mainly remained (Fig. VI-4B). In other cases, *B. cinerea* inoculation of wild type also resulted in an elimination of LM2 binding to the plasma membrane epitope (Fig. VI-4C). In *sitiens*, *B. cinerea* inoculation also caused LM2 binding intensity to stay unchanged (Fig. VI-4D) or to decrease in some tissue regions (Fig. VI-4E and 4F). However, some *sitiens* cells near *B. cinerea*-penetrated tissue regions displayed strong LM2 binding in parts of the plasma membrane (Fig. VI-4E and 4F). The abundant presence of the LM2 epitope was especially clear in the plasma membranes of *sitiens* mesophyll cells near epidermal cells showing *B. cinerea* induced anticlinal cell wall fortifications (Fig. VI-4G). For *sitiens* and for wild type, the different reactions that were observed for each genotype were all frequently present and all represented about the same proportion of the samples. No reaction was dominantly present.

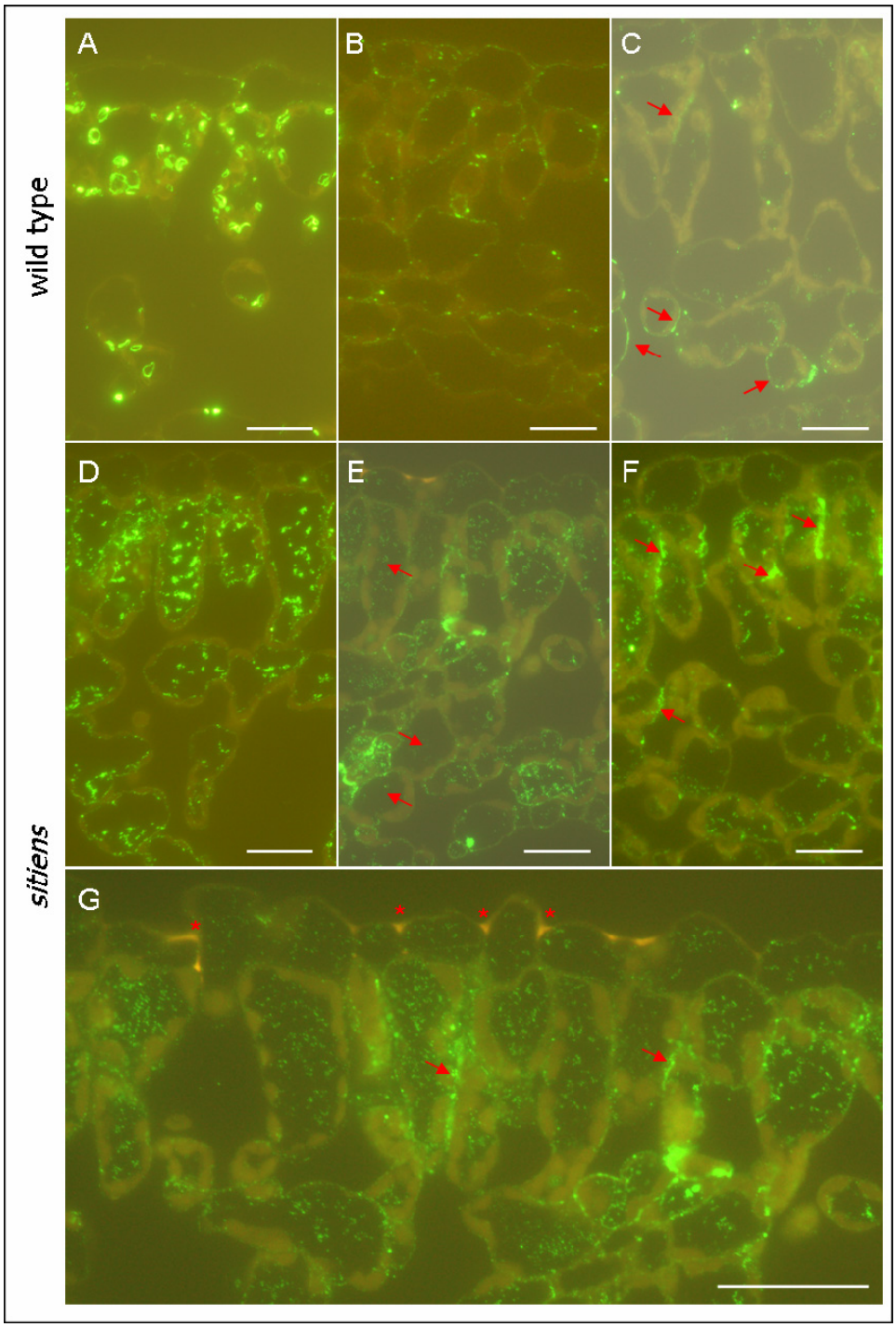

Figure VI-4: Immunolocalisation of arabinogalactan proteins with LM2 monoclonal antibody in wild-type (A-C) and *sitiens* (D-G) tomato leaves 16 h after inoculation with *B. cinerea*.
Semi-thin cross-sections of excised parts of *sitiens* and wild-type leaves that were embedded in LR white resin were incubated with LM2. Visualisation was done with epi-fluorescence microscopy after secondary labelling with FITC. At least 20 samples originating from different plants were examined for wild type and *sitiens*. Representative pictures of *sitiens* and wild type are shown.
A: LM2 epitope present in chloroplasts and plasma membrane, similar to wild-type mock-inoculated control samples (not shown); B: Loss of LM2 epitope in chloroplasts, but LM2 epitope is still present in plasma membrane; C: Loss of LM2 epitope in chloroplasts and plasma membranes. Some plasma membrane regions still contain LM2 epitopes (arrows); D: LM2 epitope present in plasma membranes and vesicle-like structures, similar to *sitiens* mock-inoculated control samples (not shown); E: Decrease of LM2 epitope presence in vesicle-like structures in some cells (arrows); F, G: Abundant presence of LM2 epitope at the surfaces of some cells (arrows), especially in tissue regions with anticlinal epidermal cell wall fortifications (asterisks). Scale bar = 50 µm.

Extensin distribution in wild-type and *sitiens* leaf tissue

We used the monoclonal antibody LM1 to compare extensin distribution and content in *sitiens* and wild-type tomato leaves, both prior to and after *B. cinerea* inoculation. LM1 recognises an epitope that is carried by a range of hydroxyproline-rich glycoproteins of the extensin class. The LM1 epitope most likely contains extensin glycan components (Smallwood et al., 1995). In wild type and *sitiens*, the LM1 epitope was mainly present in periclinal epidermal cell walls (Fig. VI-5A). No clear differences were detected between *sitiens* and wild-type in LM1 distribution or LM1 epitope quantity. In about half of the samples LM1 binding was only very weak or was completely absent. *B. cinerea* inoculation resulted in few if any changes in LM1 immunolocalisation. The LM1 epitope walls after *B. cinerea* inoculation was not present in other sites then in the outer periclinal epidermal (Fig. VI-5B). In some *sitiens* samples, there was a limited increase in LM1 binding intensity (Fig. VI-5B), but this was not consistent in all samples.

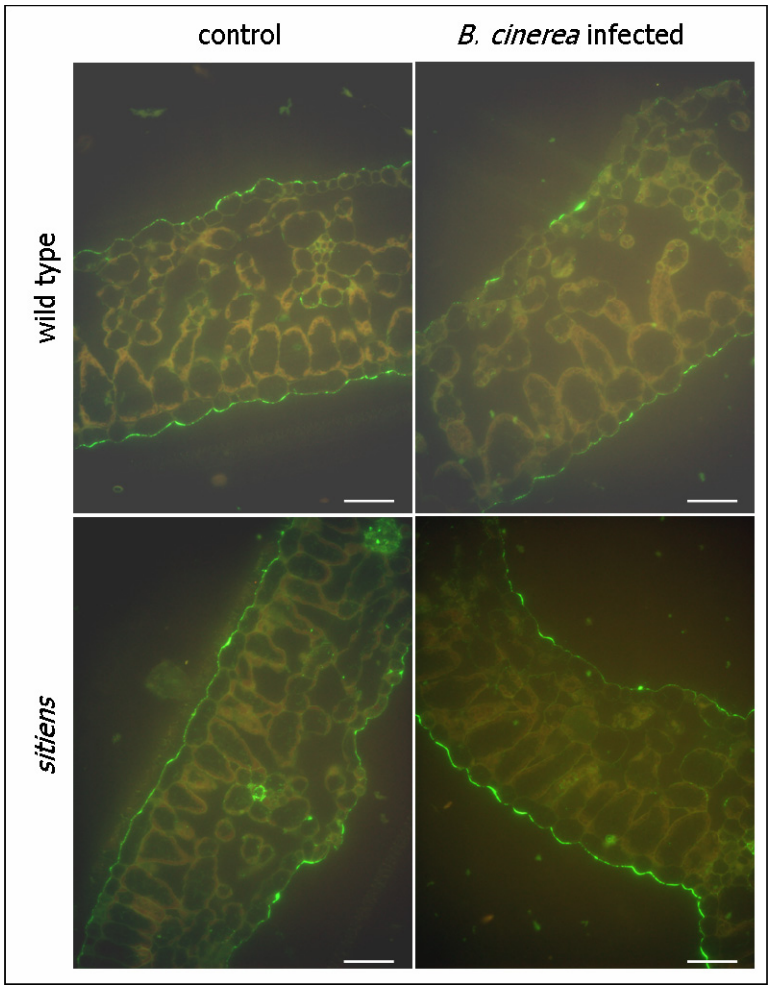

Figure VI-5: Immunolocalisation of extensin with LM1 monoclonal antibody in *sitiens* and wild-type tomato leaves before and 16 h after infection with *B. cinerea*.
Semi-thin cross sections of excised parts of *sitiens* and wild-type leaves that were embedded in LR white resin were incubated with LM1. Visualisation was done with epi-fluorescence microscopy after secondary labelling with FITC. At least 20 samples originating from different plants were examined. Some samples did not contain LM1 epitopes. Representative pictures of each sample type are shown. Scale bar = 50 µM.

DISCUSSION

We have shown that ABA deficiency in *sitiens* tomato results in enhanced and faster pathogen defence activation, resulting in increased resistance to different pathogens mediated by cell wall modifications (see chapters 2 and 5). Other data indicated that *sitiens* activates its defences upon recognition of endogenous cell wall-derived oligogalacturonide (OGA) elicitors (see chapter 5). It was hypothesised that *sitiens* has an enhanced capacity to sense pathogen-induced cell wall breakdown, leading to earlier and stronger defence activation. The data in this chapter are consistent with this hypothesis and provide new insights on possible mechanisms by which *sitiens* increases its capacity to sense perturbations in cell wall integrity and consequently enhances its defences.

Pectinases, secreted by plant pathogens, degrade homogalacturonan in the cell wall and release OGAs that are detected by the plant as signals to initiate defence responses. The polymerisation and methylation degree of the released OGA can determine the ability to trigger defence responses (Ridley et al., 2001; Barras et al., 1994). Leaves of the *sitiens* tomato mutant contain lower levels of total bulk pectic components compared to wild-type tomato, but with a slightly higher degree of methylation, resulting in about the same amount of methylated pectin and less non-methylated pectin (Katrien Curvers, personal communication; Stage report Katrien Curvers INRA – Versailles November 2006-januari 2007). Immunolocalisation experiments show that *sitiens* contains relatively less pectin in its outer periclinal epidermal cell wall than wild type. The thick layer of pectin in wild-type outer periclinal cell walls compared to the anticlinal and inner periclinal epidermal wall and to the walls in other cell types was even more pronounced after immunostaining with JIM5 (recognises both partially methylesterified homogalacturonan and un-esterified homogalacturonan) than with JIM7 (recognises partially methylesterified homogalacturonan but does not bind to un-esterified homogalacturonan). Both JIM5 and JIM7 bind to pectin with a wide range of degrees of methylesterification, and the assumption can not be made that JIM5 preferably recognises de-esterified homogalacturonans and that JIM7 is specifically recognises methylesterified homogalacturonans (Ridley et al., 2001). Therefore, our results should be interpreted with care: *sitiens* clearly contains a thinner layer of pectin in its outer periclinal epidermal walls than wild-type; whether these differences originate mainly from methylated or non-methylated galacturonans remains to be clarified. The use of monoclonal antibodies (such as JIM5 and JIM7) for immunolocalisation of specific pectin epitopes is a powerful tool to study histological and ultrastructural differences in pectin

distribution and composition. The use of more antibodies recognising specific pectin epitopes could further characterise changes in tomato cell wall composition resulting from ABA deficiency. Other examples of monoclonal antibodies that could be used are PAM1 (recognises esterified homogalacturonan; Willats et al., 1999; Mansfield et al., 2004), 2F4 (recognises calcium cross-linked pectin; Schindler et al., 1995), LM5 (recognises 1,4-linked β-D-galactan; Jones et al., 1997); LM6 (recognises 1,5-linked α-L-galactan; Jones et al., 1997), LM8 (recognises xylogalacturonan; Willats et al., 2004), LM7 (recognises non-blockwise partially methylesterified homogalacturonan; Willats et al., 2001; Clausen et al., 2003) or LM9 (recognises feroylated 1,4 linked β-D-galactan; Clausen et al., 2004). Alternatively, chemical analytical comparison of cell wall composition (by for example GC-MS) of different leaf cell types (for example on leaf epidermal strips) could confirm the differences in *sitiens* and wild-type cell wall composition on a histological level. Furthermore, experimental set-ups measuring and chemically characterising OGA release after pathogen inoculation or after different pectinolytic enzyme applications could provide more insights regarding the effect of different pectin composition on differential OGA defence elicitation in *sitiens* and wild type. Immunolocalisation of *sitiens* and wild-type pectin and pectin degradation after *B. cinerea* inoculation was also performed with JIM7 and JIM5, and was presented in chapter 3.

There are reports on the effect of plant hormones on pectin composition and biosynthesis. Some contradictory results have been obtained about the role of ABA in pectin methyl esterase (PME) regulation (Micheli, 2001). PME activity was inhibited by ABA during yellow cedar (*Chamaecyparis nootkatensis*) seed germination (Ren and Kermode, 2000), but in tomato seeds ABA increased PME activity (Downie et al., 1998). In ripening banana (*Musa* sp.) fruit, PME activity was increased by ABA application (Lohani et al., 2004). During water-deficit, known to increase plant ABA content, genome wide-gene expression studies revealed the down-regulation of the whole class of PME genes (Bray et al., 2004). Together these results indicate that ABA can influence the degree of pectin methylation by regulating PMEs. However, due to the huge complexity of both pectin biosynthesis (Ridley et al., 2001) and ABA-mediated responses (see chapter 1), no consistent or simple effects of ABA on pectin composition are available. The data in this chapter indicate that also spatial effects of differential pectin composition or content should be taken into account.

Oxidative cross-linking of extensins leads to the formation of an extensin network, which is important for the resistance to pathogen lytic enzymes (Ribeiro et al., 2006). We evaluated the immunolocalisation of extensins in *sitiens* and wild-type during *B. cinerea*

infection. No clear differences between *sitiens* and wild-type were detected and no obvious effects of *B. cinerea* inoculation were present. This negative result contrasts the strong accumulation of cross-linked proteins in the anticlinal epidermal walls at the sites of hydrogen peroxide production in *sitiens* after inoculation with *B. cinerea* (see chapter 2). The LM1 antibody was generated against rice hydroxyproline-rich glycoproteins (Smallwood et al., 1995). It is possible that the cross-linked proteins in the *sitiens* anticlinal walls are not recognised by LM1.

AGPs are important for signalling at the cell surface by linking cell wall, plasma membrane and cytoplasm, and were proposed as essential molecules in the early signalling events upon cell wall perturbations upon pathogen attack (Showalter, 2001; Knox, 2006; Seifert and Roberts, 2007). In our lab, it was shown previously with macroarray analysis that the tomato homologue of the Arabidopsis (*Arabidopsis thaliana*) fasciclin-like AGP gene *FLA9* was strongly upregulated in *sitiens* 24 h after inoculation with *B. cinerea* (Rotthier, 2004). These results were confirmed in a transcriptome analysis with TOM1 microarrays (see chapter 2). The increase in *FLA9* expression in *sitiens* 8h after *B. cinerea* inoculation was significant at $p < 0.01$, but not at $p < 0.001$ (data not shown). Interestingly, *FLA9* was also the strongest downregulated gene in Arabidopsis cellulose synthase mutants which have elevated ABA levels with a function in pathogen defence (Hernández-Blanco et al., 2007). Together these results indicate that expression of the fasciclin-like AGP *FLA9* is negatively regulated by ABA during plant-pathogen interactions.

Immunolocalisation of the LM2 AGP epitope revealed intriguing differences between *sitiens* and wild-type tomato leaves in the cellular location of AGPs. We expected to localise AGPs near sites of pathogen-induced cell wall fortification in *sitiens*. However, next to plasma membrane AGPs, LM2 mainly bound to intracellular vesicles in *sitiens* and to sites in chloroplasts in wild type. Immunolocalisation studies have appointed AGPs to the outer face of the plasma membrane, the cell wall, the endoplasmic reticulum and to multi-vesicular bodies, endomembranes, vacuoles and golgi-derived vesicles (Šamaj et al., 2000; Showalter, 2001). To our knowledge, localisation of AGP in chloroplasts has not been reported before. However, because AGPs detected with LM2 were found in tonoplasts and cytoplasmic strands (Šamaj et al., 2000), showing that some AGP epitopes are closely associated with endomembranes, it can be considered that membrane-rich organelles such as chloroplast should contain AGPs. Alternatively, cross-reactions of LM2 with the high concentrations of different sugars in chloroplasts (organelles for sugar biosynthesis) could be responsible for a false positive result.

The presence of AGPs in chloroplast of tomato leaves should be confirmed with other methods for AGP localisation, such as the use of other AGP-recognising antibodies (JIM8, JIM13, JIM14 or CCRC-M7; Seifert and Roberts, 2007) or with AGP-binding Yariv's reagent (Yariv et al., 1962). In *sitiens*, the LM2 epitope was abundantly present in vesicle-like structures. It was suggested that AGPs found in intracellular, multivesicular bodies reflect turnover of AGPs (Herman and Lamb, 1992; Showalter, 2001). The strong accumulation of AGPs in *sitiens* cell surfaces after *B. cinerea* inoculation probably reflects their involvement in cell wall-related pathogen defence. In conclusion, the abundant presence of AGPs in intracellular vesicles in *sitiens* and the accumulation of AGPs in *sitiens* cell surfaces near *B. cinerea* infected tissue regions points to a role for AGPs in the enhanced capacity of *sitiens* to activate defences upon pathogen-induced cell wall alterations.

The results presented in this chapter open up new perspectives on the possible mechanisms that allow *sitiens* plants to rapidly respond to invading pathogens that cause cell wall breakdown. Changes in architecture and composition of the pectin network and in AGP cellular distribution perhaps reflect ABA-regulated mechanisms that affect recognition and early defence signalling upon pathogen attack.

MATERIALS AND METHODS

Plant growing conditions and pathogen inoculation

Tomato (*Solanum lycopersicum* L., previously *Lycopersicon esculentum* Mill.) *sitiens* mutants (Taylor et al., 1988), provided by Prof. M. Koornneef (Wageningen University, The Netherlands) and the corresponding wild-type cultivar Moneymaker were grown in potting compost soil (Substrat 4; Klasmann-Deilmann, Groß Hesepe, Germany) at 22°C. The plants were raised in a growth chamber with 75% relative humidity in a 16 h/8 h light-dark regime. After 4 to 5 weeks, when seedlings were at the 5th leaf stage, sections excised from the tertiary leaves were used for comparisons between *sitiens* and wild type. For experiments comparing *sitiens* and wild-type reactions after inoculation with *B. cinerea*, leaf discs were punched out of the tertiary leaves with a 1-cm diameter cork bore and placed floating with the adaxial side up in 24-well plates (VWR, Leuven, Belgium). Each well was filled with 1.5 ml of distilled water. Conidia of *B. cinerea* strain R16 (Faretra and Pollastro, 1991) were obtained as previously described (Audenaert et al., 2002a). The conidial suspension was centrifuged for 10 min at 10 000x*g*. After removal of the supernatant and re-suspension of the conidia in distilled water, an inoculation suspension was prepared containing $6.25x10^4$ conidia/ml in 16.7 mM KH_2PO_4 and 25 mM glucose. Conidia were pregerminated for 2 h in the inoculation suspension at 22°C. One 5-µl droplet was used to inoculate each tomato leaf disc and incubation was done at 22°C under dark conditions. Samples were taken after 8, 12, 16, 20, 24, 32, 48 and 72 hpi. Microscopy on infected samples was always compared with mock-inoculated samples (inoculated with a droplet containing only 16.7 mM KH_2PO_4 and 25 mM glucose). Differences in *B. cinerea* symptoms in *sitiens* and wild-type were confirmed by evaluation after 4 days. Each inoculation droplet was classified as a spreading or non-spreading lesion and the data were analyzed with a binary logistic regression.

Immunological staining procedures

Cross-sections of tomato leaf sections embedded in LR White resin (LRW; Sigma-Aldrich, Fluka) were used for all immunological staining experiments. Leaf segments (2 mm × 8 mm) were excised and fixed in 4% paraformaldehyde and 1% glutaric dialdehyde (Acros organics) in 0.05M PIPES (piperazine N,N'-bis(2-ethane sulfonic acid) disodium salt; Sigma-Aldrich) for 3 hours with the first 30 min under vacuum pressure. Four 30 min washing steps were done in PIPES. Dehydration steps of 30 min were performed with successive increasing ethanol concentrations (30%, 50%, 70% and 95% respectively). Addition of LRW to the samples was done gradually: 1/3 LRW + 2/3 ethanol for 40 min; 1/2 LRW + 1/2 ethanol for 40 min and 2/3 LRW + 1/3 ethanol for 40 min. Next, three successive incubations of 2 h in pure LRW were done. The samples were transferred to electron-microscopy-purpose beem micro embedding capsules (Laborimpex) filled with LRW and incubated for 24 h at 60°C for polymerisation. Semi-thin sections (2 μm) were cut with a Leica RM2265 motorised rotary microtome (Leica Microsystems, Nussloch, Germany) transferred to adhesive object slides (Klinipath). A subset of the samples was stained with toluidine blue to allow topographic visualisation of chloroplasts and cell walls. For immunological staining, small volumes (80-150 μl) of the solutions were placed on the region of the objectives slide containing the sections, which was surrounded by a hydrophobic coating placed with a PAP pen (Sigma-Aldrich). Immunological staining was done by incubation in TBSB-T (Tris-buffered saline solution pH 7.4 + 0.1% bovine serum albumin (BSA) + 0.01% Tween-20) for 5 min, followed by a 20 min pre-incubation step in 3.33% normal rabbit serum (Gentaur) as a blocking solution in TBSB-T. Incubation in the primary antibody, 20 times diluted in TBSB-T, was done at 4°C for 16 h. The following monoclonal primary antibodies produced in rat were used: JIM5, JIM7, LM1 and LM2 (PlantProbes, UK). Subsequently, samples were washed three times in TBSB (without tween) for 10 min and incubated in the secondary antibody: 1 h incubation in anti-Rat IgG (whole molecule) – fluorescein isothiocyanate (FITC) antibody produced in rabbit (Sigma-Aldrich) in the dark. After six successive 10 min washing steps in the dark, samples were mounted in Vectashield Hardset mounting medium (VWR) and examined with a Olympus BX51 epi-fluorescence microscope with U-MWB2 filter cube (450-480 nm excitation filter, DM 500 dichroic beam splitter and BA515 long-pass filter). For every antibody, negative controls were used, consisting of removal of primary and secondary antibody, to verify the antibody specificity.

Chapter

7

Conclusions and General Discussion

ABA-deficiency primes tomato for pathogen defence responses

In response to the environment, ABA is primarily an abiotic stress hormone: drought, cold or salt stresses induce large increases in plant ABA concentrations and activate ABA-responsive signalling pathways, resulting in a range of stress-protective measures. During biotic stress ABA can negatively affect defence responses, which is supported by the results obtained in this work. The contrasting function of ABA in the response to abiotic and biotic stresses is beautifully exemplified in the ABA-deficient *sitiens* tomato (*Solanum lycopersicum*) mutant. Whereas *sitiens* is extremely vulnerable to abiotic stresses and fails to cope with changes in environmental conditions (such as temperature or relative humidity; Nagel et al., 1994), *sitiens* has an enhanced capacity to resist pathogens. The *sitiens* mutant was shown to be more resistant than wild-type tomato to *Botrytis cinerea* (Audenaert et al., 2002a; see chapter 2) *Oidium neolycopersici* (Achuo et al., 2006), *Pseudomonas syringae* (Thaler and Bostock 2004), *Erwinia chrysanthemi* (see chapter 5) and *Sclerotinia sclerotiorum* (Rotthier, 2004). This work has shown that *sitiens* is in a pre-alerted state of defence. The enhanced capacity of plants to mobilize infection-induced cellular defence responses is often referred to as "priming". The primed state of *sitiens* was evaluated by gene expression and biochemical analysis. Transcriptome analysis with tomato TOM1 microarrays revealed a basal upregulation of pathogen defence genes, which were even higher expressed rapidly upon challenge with *B. cinerea* (see chapter 2). Similarly, peroxidases had higher basal levels of activity in *sitiens* than in wild type, and peroxidases were further activated after challenge with *B. cinerea* or *E. chrysanthemi* (see chapters 2 and 5). In addition, *sitiens* was previously shown to be hyper-susceptible to BTH-induced resistance (Audenaert et al., 2002a). In general, it seems that *sitiens* is primed for pathogen defence, resulting not only in an earlier activation, but also in defence reactions that are more extensive and larger in amplitude than wild-type tomato plants. This work supports other findings that indicate that high or basal ABA levels restrain defence responses, whereas ABA-deficiency leads to a hyperinduction of pathogen defence responses (see chapter 1).

Hydrogen peroxide accumulation and cell wall fortification are essential for *sitiens* resistance, whereas ABA-responsive callose deposition is involved in basal tomato defences

The defence reactions of *sitiens* and wild-type plants were evaluated by using different methods and experimental set-ups, including transcriptome analysis, biochemical characterisation and histochemical and immunological microscopy analysis. *Sitiens* defence responses were characterised by a very early and strong accumulation of hydrogen peroxide at the site of *B. cinerea* and *E. chrysanthemi* infection. The accumulation of hydrogen peroxide was shown to be essential in establishing resistance in *sitiens*, because enzymatic or antioxidant hydrogen peroxide removal or blocking hydrogen peroxide production resulted in restoration of susceptibility. Production of hydrogen peroxide and other reactive oxygen species (ROS) during a so-called oxidative burst are important mediators of biotic stress responses and can have diverse functions in conferring resistance, such as direct antimicrobial activity, acting as defence signalling molecules and leading to cell wall fortification and induction of the hypersensitive response (HR) (Lamb and Dixon, 1997). It is remarkable that extensive ROS accumulation, as detected in *sitiens*, leads to resistance to the necrotrophic pathogens *B. cinerea* and *E. chrysanthemi*, because the oxidative burst was shown to be only effective against biotrophic pathogens, whereas it has no effect towards necrotrophs, or even helps these type of pathogens in colonising host tissue (Glazebrook, 2005; Govrin and Levine, 2000; De Vleesschauwer et al., 2006). It was shown that defence-related ROS-formation leading to HR cell death is exploited by *B. cinerea* as a pathogenicity factor (Govrin and Levine, 2000). In *sitiens*, hydrogen peroxide accumulation was specifically detected in anticlinal walls of epidermal cells near *B. cinerea* inoculated tissue regions, where it led to cell wall fortification by protein cross-linking and incorporation of phenolic compounds. The role of cell wall fortification in *sitiens* defence was confirmed by higher expression of genes involved in biosynthesis of cell wall phenolic compounds through the phenylpropanoid pathway and biosynthesis of structural cell wall proteins. Microscopical analysis of pathogen progress in *sitiens* leaf tissue showed that both *E. chrysanthemi* (visualised directly as intercellular micro-colonies) and *B. cinerea* (visualised indirectly as progress of pectin degradation) were arrested by hydrogen peroxide-induced cell wall fortification. Negative effects of ABA on cell wall fortification were recently confirmed in Arabidopsis interacting with *Pseudomonas syringae* (Mohr and Cahill, 2007).

The contribution of callose deposition to wild-type and *sitiens* defence responses was also evaluated. The *sitiens* mutant showed a decrease in the amount of callose deposited after *B. cinerea* inoculation compared to wild-type tomato and callose deposition did not significantly contribute to the resistant response in *sitiens*. Basal ABA levels in wild-type plants allowed more callose deposition, which in addition was implicated in the establishment of basal defences. These results are consistent with a number of reports in various plant-pathosystems showing a positive correlation between ABA and callose deposition (Ton and Mauch-Mani, 2004; Kaliff et al., 2007; Adie et al., 2007; Wiese et al., 2004; Flors et al., 2005).

Together these results indicate that ABA-deficiency in tomato leads to the hyperinduction of a specific set of pathogen defence responses, including extracellular ROS formation, cell wall fortification and SA-responsive defence signalling, at the expense of pathogen-induced callose deposition (and responses to abiotic stresses). The type of defences that are strongly activated in *sitiens*, are normally associated with the response to biotrophic pathogens and are considered to be less effective against necrotrophs (Thomma et al., 1998; Glazebrook, 2005). The effectiveness of these responses towards *B. cinerea* and *E. chrysanthemi*, two pathogens considered to have a strictly necrotrophic lifestyle, shows the hyperinduction in *sitiens* of these specific defence responses and is indicative of the general shift towards pathogen defence at the cost of other (abiotic) stress responses.

How does ABA-deficiency cause priming for pathogen defence responses?

It is clear that *sitiens* mutants have an enhanced defensive capacity to resist pathogen attack. Furthermore, we have shown that fast and extensive hydrogen peroxide-mediated cell wall modification is a key event in restriction of *B. cinerea* and *E. chrysanthemi*. One of the main and fundamental challenges is to reveal the mechanisms by which ABA-deficiency leads to priming for pathogen defence. Several possible links between biotic stress responses and the functions of ABA in abiotic stress responses or developmental processes were proposed and discussed in this thesis. It is essential to mention that all of these ABA – biotic stress response connections are not per se mutually exclusive. Moreover, as ABA levels appear to control the general shift in priority of response to either biotic or abiotic stress (see chapter 1), decreased ABA levels will allow plants to simultaneously apply multiple strategies to prioritise biotic stress defences, which is consistent with the function of ABA as a multi-component regulator in other processes.

One interpretation is that the *sitiens* mutant can be considered to permanently suffer from drought stress because of the lack of ABA-mediated stomatal regulation (Nagel et al., 1994), which might explain the higher level of pathogen defences in the mutant, because abiotic stress can cause priming for pathogen defence (Conrath et al., 2002). Several ABA-signalling components are implied in biotic stress responses. Ca^{2+} signalling is important in disease resistance and in ABA-responsive guard cell signalling and dehydration responses (Mauch-Mani and Mauch, 2005). Similarly, ROS homeostasis and signalling is controlled by ABA during stomatal movements and in response to abiotic stresses and is essential for disease resistance during the oxidative burst. This work has shown that ABA-deficiency in tomato leads to stronger and more rapid ROS-formation upon pathogen attack and contributes in establishing the complex interaction between ROS and ABA during various types of stresses. Also the regulation by ABA of the phenylpropanoid biosynthetic pathway can help to explain the negative effects of ABA on disease resistance. Phenylpropanoid biosynthesis results in formation of antimicrobial secondary metabolites, components required for cell wall fortification and synthesis of the defence hormone SA. This work has shown that ABA not only downregulates phenylpropanoid biosynthesis through transcriptional repression of PAL, as was shown previously (Ward et al., 1989; McDonald and Cahill, 1999; Audenaert et al., 2002a), but also by transcriptional repression of other enzymes in the pathway (see chapter 2), which was confirmed in other recent studies (Mohr and Cahill, 2007). Otherwise, Pti5, a tomato pathogen-inducible ethylene response element-binding protein-like transcription factor, whose expression is higher in *sitiens* than in wild type prior to pathogen inoculation, can provide a link between ABA deficiency and pathogen-induced gene activation. Pti5 is expressed specifically during biotic but not abiotic or hormonal stresses (Thara et al., 1999) and regulates SA-induced PR protein genes (Gu et al., 2002), suggesting a specific role for Pti5 in plant defence against pathogens. Hence, high expression levels of PR proteins in *sitiens* before and after *B. cinerea* inoculation might be a result of Pti5-mediated regulation. Also, reduction of ABA-induced callose deposition in *sitiens* can have a positive effect on pathogen defence. ABA primes for callose deposition (Flors et al., 2005), which in its turn can block SA-inducible defence responses (Nishimura et al., 2003).

We have shown that *sitiens* defence responses are probably mainly activated upon recognition of endogenous cell wall elicitors (see chapter 5). In chapter 6 we explore the hypothesis that ABA-regulated structural changes in cell wall composition possibly enhance *sitiens* defence activation. Spatial and chemical changes in pectin structure and abundant presence of arabinogalactan proteins in vesicles are possible mechanisms that allow ABA-

deficient plants to sense pathogen-induced cell wall alterations faster and subsequently activate their defences. Collectively, we have proposed several putative mechanisms that explain enhanced priming for pathogen defence resulting from ABA deficiency. Further research will be needed to determine their contribution to resistance in *sitiens*.

Implications for the view on defence against necrotrophic pathogens

Plant defence against necrotrophic pathogens is believed to be different from defence against biotrophic pathogens. Necrotrophic pathogens are typically characterised by having a broad host range, i.e. only few plant species display non-host resistance, and there is generally no R-gene-mediated resistance against necrotrophic pathogens. As a result, resistance against necrotrophs is scarce, which is especially the case for *B. cinerea* and *E. chrysanthemi*, two pathogens with a strictly necrotrophic lifestyle. Therefore, the high level of resistance to these pathogens in *sitiens* merits our attention.

It was suggested that defence against biotrophic pathogens usually depends on SA-responsive defence signalling, whereas defence against necrotrophic pathogens is predominantly mediated by the synergistic action of JA and ET (Thomma et al., 1998; Glazebrook, 2005). However, the interaction of tomato with the necrotroph *B. cinerea* was shown to be an exception to this model, with defence responses depending on SA signalling (Audenaert et al., 2002a; Achuo et al., 2004). Furthermore, it was shown that tomato ABA deficiency results in a hyperinduction of SA-dependent defence responses (Audenaert et al., 2002a). In this work we show that extensive hydrogen peroxide accumulation in *sitiens* is essential in conferring resistance to *B. cinerea*. ROS formation during an oxidative burst is generally considered to be effective against biotrophs, but not against necrotrophs. Moreover, it was shown that an oxidative burst is exploited by necrotrophic pathogens, including *B. cinerea*, which utilise ROS-induced programmed cell death to colonise host tissue (Govrin and Levine, 2000; De Vleesschauwer et al., 2006). We add some refinement to this controversial topic and suggest that timing, localisation and function of ROS accumulation determine its effect on the outcome of the interaction with a necrotrophic pathogen. As a result, our data indicate that ROS-dependent defence responses that are typically involved in defence against biotrophic pathogens are also very effective in arresting necrotrophic pathogens when these are hyperinduced, as is the case in the *sitiens* mutant.

Implications for cross-talk between biotic and abiotic stress responses

Modern agriculture has to cope with an increasing number of environmental constraints. Restriction of chemical protection treatments in integrated crop management makes farmers more dependable on disease control measures that rely on natural plant defence mechanisms, such as induction of resistance and resistance breeding. In addition, worldwide economical losses caused by salt, temperature and drought stress are huge, and are believed to increase further in the future as a result of climate evolution, leading also in Europe to higher temperatures, water shortage and desertification. Efforts from plant breeders to improve crop quality no longer fixate mainly on increasing yield, but focus on improving abiotic stress tolerance and biotic stress resistance. Fundamental knowledge on how plants cope with the regulation and coordination of responses to different stresses is essential to engage in these challenges. ABA has a well studied role in response to abiotic stresses, but its key function in response to pathogens has long been a neglected field of research. The results obtained in this work add to the growing body of evidence that implicate ABA as a global coordinator of abiotic and biotic stress responses.

It is clear that alterations in endogenous ABA levels change the plant's defensive capacity towards abiotic and biotic stresses. ABA deficiency in *sitiens* results in enhanced pathogen defence responses at the expense of reduced abiotic stress tolerance resulting from impaired regulation of plant water relations. In other words, ABA levels control a global shift in the priority to react to a certain stress. These findings receive important support when we consider plants in their natural environment. In nature, abiotic stresses such as drought stress do not frequently coincide with pathogen attack, as plant pathogens generally require humid conditions to cause disease. This justifies the existence of a global switch to react to either drought stress or to pathogen attack. Another important aspect is that abiotic stress (drought stress) in general imposes a greater threat to plant survival than biotic stress. Abiotic stresses that disturb plant water balances and fluxes require immediate (ABA-mediated) action before irreversible wilting takes place. This is consistent with the dominant nature of ABA-dependent responses over biotic stress response signalling, which was frequently reported (Thaler and Bostock, 2004; Andersen et al., 2004; Fujita et al., 2006). Taken together, it seems that changes in ABA levels are a powerful tool of plants to mount abiotic stress protective measures and at the same time control the activation and amplitude of biotic stress responses.

An important question is how general the suppressive effect of ABA on pathogen

defence really is. Do ABA levels control the activation and/or amplitude of pathogen defence responses in the majority of plant-pathogen interactions? Measurement of ABA during the early stages of pathogen infection reveals that ABA levels increase in compatible interactions, but not in incompatible interactions (Cahill and Ward, 1989). The lack of available data that includes ABA measurements for a wider range of plant-pathosystems hinders the formation of general conclusions regarding the evolution of plant ABA levels during resistant or susceptible responses. However, important information can be derived from the function of ABA as a virulence factor of bacterial and fungal plant pathogens. Many fungi associated with plants produce ABA, and the involvement of fungal ABA in suppressing defence was suggested (Kettner and Dörffling, 1991; see chapter 1). Moreover, fungi and bacterial pathogens were shown to stimulate ABA signalling as an essential strategy to cause disease (de Torres-Zabala et al., 2007). In addition, there are convincing findings that indicate that ABA also suppresses defence responses during symbiotic interactions of plant roots with arbuscular mycorrhizal (AM) fungi and nitrogen-fixating bacteria (see chapter 1). Together these findings suggest that ABA-mediated suppression of pathogen defence responses is a wide-spread phenomenon which controls the outcome of many plant-microbe interactions.

Tomato is useful as a model plant

Using tomato as a host plant to study plant-pathogen interactions has a number of important advantages. First, tomato is a cultivated crop plant, which suggests that a large proportion of its capacity to synthesise antimicrobial secondary metabolites has been lost through a history of selection for increased productivity and yield. Weeds such as the model plant *Arabidopsis thaliana* are still adapted to survival in natural environments and carry a number of constitutive defences, resulting in high levels of non-host resistance to various pathogens. Hence, tomato is a better host than Arabidopsis to study the contribution of inducible plant defences in the establishment of resistance. Secondly, tomato is a model plant to study wound responses and responses to abiotic stresses (Bowles, 1998; Wasternack et al., 2006), allowing a meaningful study of stress response interactions. Third, tomato is a member of the *Solanaceae* plant family including other economical important crops such as potato (*Solanum tuberosum*), tobacco (*Nicotiana tabacum*), petunia (*Petunia hybrida*), pepper (*Capsicum annuum*) and eggplant (*Solanum melongena*). Tomato is tolerant to inbreeding, the tomato genome is currently being sequenced and several biotechnological tools for tomato are available (Sol Genomics Network, http://www.sgn.cornell.edu/), together making tomato the

genetic centrepiece and the model plant of the economically important *Solanaceae* family.

Perspectives and directions for future research

Although several conclusions could be drawn from this work, important questions still need further experimental investigation. In addition, this work has opened up several perspectives for further research.

∴ We have characterized *sitiens* defence responses by use of immuno- and histochemical microscopy and by a transcriptome study after pathogen inoculation, and found evidence for the participation of cell wall fortification and defence gene expression. However, it can not be excluded that other types of biochemical defence responses are also involved in the resistant interaction. Further analysis of *sitiens* defence responses could employ a proteomic or metabolomic comparison of *sitiens* and wild-type tomato after pathogen challenge.

∴ ABA-responsive plant processes are complex in nature, involving multi-component signalling pathways, multiple ABA perception sites and ABA-concentration dependent processes (see chapter 1). Evaluation of spatial and temporal evolution of ABA levels by organ- and cell-specific time-course ABA measurements should provide more information on concentration-dependent ABA action during plant-pathogen interactions. Furthermore, evaluation of disease resistance and defence responses in other ABA-biosynthetic and ABA-signalling tomato mutants can help to decipher the role of ABA in tomato defence responses. For example, the ABA overexpression transgenic tomato lines recently developed by Thompson et al., (2007) and lines touched in homologues of known Arabidopsis ABA signalling genes (see Finkelstein and Rock, 2002), could be employed.

∴ It was shown that ABA-deficient mutants are more resistant than wild-type tomato to the pathogens *Botrytis cinerea* (Audenaert et al., 2002a; see chapter 2) *Oidium neolycopersici* (Achuo et al., 2006), *Pseudomonas syringae* (Thaler and Bostock 2004), *Erwinia chrysanthemi* (see chapter 5) and *Sclerotinia sclerotiorum* (Rotthier, 2004). Testing more pathogens for their capacity to infect ABA-deficient mutants would expand our knowledge. Furthermore, comparison of the defence responses influenced by ABA in each interaction should provide more information on the range

of pathogens affected by the defence responses described in this work. From a broader perspective, it could be investigated whether ABA-dependent defence responses are also involved in other types of resistance in tomato. For example, it was shown that BTH and several *Pseudomonas* strains induce resistance to *B. cinerea* in tomato (Audenaert et al., 2002a; Audenaert et al., 2002b). Evaluation of the role of ABA and of the defence responses observed in *sitiens* in these forms of induced resistance could establish more firmly the generalisation of the results obtained in this work.

∴ The action of ABA in plant resistance can be partly attributed to its interaction with other plant defence hormones such as SA, JA and ET (Mauch-Mani and Mauch, 2005). Simultaneous measurement of plant hormones and monitoring the expression of marker genes for known plant hormone defence signalling could provide more insight in tomato hormone interactions during disease resistance. Furthermore, as mutant or transgenic lines affecting JA, ethylene or SA levels or signalling are available for tomato (Audenaert et al., 2002a), double mutants in ABA-deficient mutant backgrounds can be derived relatively straightforward.

∴ Hydrogen peroxide accumulation is an essential component for the establishment of resistance in ABA-deficient tomato (see chapters 2 and 5). ROS-generating mechanisms have been characterised, of which cell wall peroxidases and membrane bound NADPH oxidases are considered of main importance during an oxidative burst (Bolwell and Wojstaszek, 1997). Inhibitor studies blocking specific ROS-generating enzymes, organelle-specific activity measurements of enzymes involved in controlling ROS homeostasis and monitoring the expression of ROS-responsive and ROS-generating genes can reveal the mechanisms that allow ABA-deficient tomato plants to hyperinduce hydrogen peroxide-dependent defences and should provide more insights on the action of ABA upstream of ROS generation.

∴ Gene expression analysis with TOM1 microarrays has revealed a number of genes possibly involved in disease resistance that are deregulated before and quickly after pathogen challenge in resistant *sitiens* plants (see chapter 2). At a first stage, a selection of these genes will be silenced by using virus-induced gene silencing (VIGS) in *sitiens* and wild-type tomato background and the level of resistance to *B. cinerea* will be evaluated in the silenced lines. This analysis is currently being performed in our laboratory. At a second stage, a selection of the target genes that affect pathogen

resistance will be knocked out and overexpressed by using *Agrobacterium*-mediated transformation. Functional analysis of these lines should include evaluation of disease resistance and molecular, biochemical and microscopical analysis of pathogen defence responses. Among the genes deregulated in *sitiens* defence responses, a number of target genes have not been linked with pathogen defence before and open up new perspectives to study uncharacterised resistance mechanisms. Genes involved in amino acid metabolism such as the genes encoding an arginase, a bifunctional lysine-ketoglutarate reductase/saccharopine dehydrogenase and a β-alanine-pyruvate aminotransferase are among the target genes possibly involved in defence signalling and priming for pathogen defence. Moreover, these results were directly responsible for the creation of a new (FWO) project in cooperation with the Vrije Universiteit Brussel (VUB) - Faculty of Science – Department of Biotechnology entitled "role of amino acid metabolism and peroxisomal enzymes involved in disease resistance".

∴ Spatial and chemical changes in pectin structure and abundant presence of arabinogalactan proteins in vesicles are possible mechanisms that allow ABA-deficient plants to sense pathogen-induced cell wall alterations faster and subsequently activate their defence responses (see chapter 6). A further analysis of the effect of ABA on cell wall composition by immunolocalisation or chemical analysis of cell wall components could provide more insights on the possible role of ABA mediating cell wall changes responsible for differential defence activation. Moreover, a comparison between *sitiens* and wild-type tomato plants of the cell wall breakdown products released during the early stages of necrotrophic pathogen infection and of the defence activation after exposure to these breakdown products should give more information on the effect of ABA on defence elicitation.

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Summary

In order to cope with the constant threat of a wide range of potentially harmful micro-organisms, plants have developed an impressive constitutive and inducible defensive machinery of enormous complexity to combat pathogen invasion. Plant hormones are not only important for controlling plant development, but are also essential to regulate plant responses to the environment. The plant hormones salicylic acid (SA), jasmonate (JA) and ethylene (ET) are classically associated with plant pathogen defence responses as their increase in concentrations upon pathogen recognition, which leads to the activation of specific signalling cascades and pathogen defence gene expression, is important for resistance. In contrast, the plant hormone abscisic acid (ABA) has a well-established function in activating plant responses to abiotic stresses (such as cold, drought and salinity) by regulating stomatal aperture and by activating stress-responsive genes, but the effect of ABA on plant-pathogen interactions has long been a neglected research topic. Although exceptions exist, basal or high ABA levels are in most cases associated with susceptibility, while reduction of plant ABA levels often leads to increased resistance. However, our knowledge on the mechanisms of ABA-induced susceptibility is still very scarce and fragmentary.

In the present work, we have explored the influence of ABA on the defence responses of tomato towards biotic stress. We have shown that ABA deficiency in the *sitiens* tomato mutant results in increased resistance towards the necrotrophic fungus *Botrytis cinerea* and the necrotrophic bacterium *Erwinia chrysanthemi*, two pathogens for which the occurrence of resistance is very rare. Comparison of gene expression in *sitiens* and wild-type tomato with TOM1 microarrays revealed that defence-related transcript accumulation prior to infection is higher in *sitiens* than in wild type. Moreover, further elevation of defence gene expression after pathogen attack is also stronger in *sitiens*, both in number of genes and their expression levels. These results show that ABA-deficiency results in priming for pathogen defence. Annotation of the genes differentially regulated between in *sitiens* and wild type showed elevated expression levels in *sitiens* of genes encoding SA-inducible PR proteins and of genes involved in SA biosynthesis, which confirmed earlier findings that ABA deficiency leads to hyperinduction of SA-dependent defence responses.

Thorough analysis of the defence reactions that were hyperactivated in *sitiens* upon inoculation with *B. cinerea* and *E. chrysanthemi* revealed an essential role for hydrogen peroxide accumulation in the defence response towards both pathogens. Compared to wild-type

tomato, hydrogen peroxide accumulation was earlier and more extensive in *sitiens*. The necessity of this defence reaction in the establishment of resistance in *sitiens* was demonstrated by disruption of the early and strong hydrogen peroxide accumulation. Removal of hydrogen peroxide with the antioxidants catalase and ascorbate and blocking hydrogen peroxide production with diphenilene iodonium increased the levels of susceptibility in *sitiens*. Accumulation of reactive oxygen species (ROS), such as hydrogen peroxide, during pathogen attack is a well-known phenomenon and its role in arresting biotrophic pathogens is firmly established. However, ROS accumulation is supposed to have a negative effect on defence against necrotrophic pathogens such as *B. cinerea*, since elevation of *in planta* ROS levels sets of a hypersensitive response, leading to increased plant tissue colonisation by necrotrophic pathogens. Our results clearly oppose the generally accepted theorem that plant defence-related ROS formation aids necrotrophs in their pathogenicity, and show that a timely hyperproduction of hydrogen peroxide is efficient in protecting the *sitiens* tomato mutant against necrotrophic pathogen attack.

Although ROS can have other roles in defence, such as functioning as a signalling intermediate, having a direct antimicrobial effect or leading to a hypersensitive response, we have demonstrated that hyperaccumulation of hydrogen peroxide in *sitiens* leads to cell wall fortification, which results in containment of *E. chrysanthemi* and *B. cinerea*. Hydrogen peroxide accumulation in *sitiens* was accompanied by earlier and increased activation of extracellular peroxidases and a strong histochemical-detected cell wall fortification at the site of pathogen attack. These findings were consistent with the immediate hydrogen peroxide-fuelled peroxidative cross-linking of structural cell wall proteins and peroxidative incorporation of phenolic compounds during an oxidative burst. Furthermore, analysis of the genes activated in *sitiens* during *B. cinerea* inoculation revealed an overrepresentation of genes involved in cell wall modification. Microscopical analysis of pathogen progress in *sitiens* leaf tissue showed that both *E. chrysanthemi* (visualised directly as intercellular micro-colonies) and *B. cinerea* (visualised indirectly as progress of pectin degradation) were arrested by hydrogen peroxide-induced cell wall fortification. The site of pathogen arrest, i.e. the site of cell wall fortification, differed for *E. chrysanthemi* and *B. cinerea*, which was at least partly due to the nature of each inoculation procedure. *B. cinerea* penetrates the adaxial leaf cuticula and outer epidermal cell wall and was blocked by wall fortifications of the anticlinal epidermal *sitiens* cells. The arrest of *E. chrysanthemi*, which is infiltrated in the leaf tissue, was located at the sites of *sitiens* cell wall fortification at the border of the infiltration zone.

Deposition of callose at the site of pathogen entry was previously demonstrated to be influenced by ABA. We have shown that callose deposition after *B. cinerea* inoculation is weaker in *sitiens* compared to the wild type. Inhibition of callose synthesis with 2-deoxy-D-glucose did not affect resistance in *sitiens*, but caused additional susceptibility in wild type. These findings indicate that callose deposition is not part of *sitiens* defence responses that are effective in blocking *B. cinerea* and suggest that callose deposition only contributes to wild-type tomato basal resistance.

There are strong indications that the rapid and powerful defence responses in *sitiens* are triggered by recognition of endogenous plant cell wall elicitors. Both *E. chrysanthemi* and *B. cinerea* trigger the same type of defence responses in *sitiens*, which conflicts with the involvement of pathogen-specific elicitors. In addition, both pathogens produce great amounts of cell wall-degrading enzymes (CWDEs) and rely largely on cell wall pectin decomposition for their virulence. We have also shown that type II secretion-negative *E. chrysanthemi* mutants, which are incapable of secreting CWDEs, fail to fully activate *sitiens* defence responses. Furthermore, defence responses are elicited by bacteria-free CDWE-containing *E. chrysanthemi* culture filtrate. Since we found no differences between *sitiens* and wild-type in the capacity to resist *E. chrysanthemi* cell wall degradation, we have hypothesised that changes in *sitiens* cell wall composition could be responsible for differences in the release of cell wall oligomers, which are known and potent elicitors of defence responses upon pathogenic cell wall degradation. Alternatively, enhanced sensing of these oligomers in *sitiens* could also be responsible for the faster activation of defence. We found support for both hypotheses, as immunolocalisation of different cell wall components demonstrated differences between *sitiens* and wild-type in pectin distribution and in the presence of arabinogalactan proteins, which are presumably involved in signalling at the cell surface. Further experimental data will be needed expand our knowledge *sitiens* defence elicitation.

In conclusion, we have shown that ABA-deficiency in tomato results in priming for pathogen defence responses and that a rapid hyperinduction of ROS-dependent cell wall fortification is a powerful defence strategy to stop the necrotrophic pathogens *B. cinerea* and *E. chrysanthemi*.

Samenvatting

Om aan de constante dreiging van een brede waaier aan potentieel schadelijke micro-organismen te weerstaan hebben planten een indrukwekkende constitutieve en induceerbare afweer van onwezenlijke complexiteit ontwikkeld om invasie van pathogenen te verhinderen. Plantenhormonen zijn niet enkel belangrijk voor de de groei en ontwikkeling van de plant, maar zijn eveneens essentieel om de reacties van de plant op zijn uitwendige omgeving te controleren. De plantenhormonen salicylzuur (SA), jasmijnzuur (JA) en ethyleen (ET) zijn typisch geassocieerd met plantafweer tegen pathogenen. De concentratie van deze hormonen stijgt na herkenning van de pathogeen en dit leidt tot de activatie van specifieke signaaltransductiewegen en expressie van afweergenen tegen pathogenen. Daarentegen heeft het plantenhormoon abscisinezuur (ABA) een goed gekende functie in de activatie van plantreacties tegen abiotische stress-factoren (zoals droogte-, koude- en zoutstress) omdat het stomatale opening en de activatie van de stress-gevoelige genen controleert. Onderzoek naar het effect van ABA op de interactie van planten en pathogenen wordt echter reeds lang verwaarloosd. Hoewel er uitzonderingen gekend zijn, worden basale of verhoogde ABA gehaltes meestal geassocieerd met een verhoogde gevoeligheid voor pathogenen, terwijl een verlaging van ABA gehaltes vaak resulteert in verhoogde resistentie. De kennis met betrekking tot de mechanismen voor ABA-geïnduceerde gevoeligheid is echter uiterst beperkt en onvolledig.

In het huidige werk hebben we de invloed bestudeerd van ABA op de afweer van tomaat tegen biotische stress. We hebben aangetoond dat ABA deficiëntie in de *sitiens* tomatenmutant resulteert in een verhoogde resistentie tegenover de necrotrofe schimmel *Botrytis cinerea* en de necrotrofe bacterie *Erwinia chrysanthemi*, twee pathogenen waartegen resistentie slechts zelden voorkomt. Een vergelijking van genexpressie in *sitiens* en wild type tomaat met behulp van TOM1 microarrays toonde aan dat zonder pathogeeninfectie de accumulatie van afweergerelateerde transcripten hoger was in *sitiens*. Daarenboven vertoonde *sitiens* ook na pathogeeninoculatie een verhoogde stijging van expressie van afweergenen, zowel op het gebied van het aantal genen als hun expressieniveas. Deze resultaten tonen aan dat ABA deficiëntie resulteert in *priming* voor pathogeenafweer. Annotatie van de genen die een verschillende expressie vertoonden voor *sitiens* en wild type onthulde verhoogde expressieniveaus in *sitiens* voor genen die coderen voor SA-induceerbare PR-proteïnen en voor genen betrokken in SA biosynthese. Dit bevestigde onze vroegere bevinding dat ABA deficiëntie leidt tot een hyperinductie van SA-afhankelijke afweer.

Een grondige analyse van de afweerreacties die hypergeactiveerd waren in *sitiens* na inoculatie met *B. cinerea* en *E. chrysanthemi* onthulde dat de accumulatie van waterstofperoxide essentieel is voor een succesvolle afweer tegen beide pathogenen. In vergelijking met wild type tomaat vertoont *sitiens* een snellere en sterkere waterstofperoxide accumulatie. Deze accumulatie is nodig om resistentie te verkrijgen in *sitiens*, omdat een verwijdering van waterstofperoxide met de antioxidanten catalase of ascorbaat, of een verhindering van waterstofperoxideproductie met diphenileen iodonium de gevoeligheid in *sitiens* verhoogde. Accumulatie van reactieve zuurstofvormen (ROS) zoals waterstofperoxide tijdens een pathogeenaanval is een bekend fenomeen en de werking hiervan bij het bestrijden van biotrofe pathogenen staat vast. Daarentegen wordt algemeen aanvaard dat accumulatie van ROS de afweer tegen necrotrofe pathogenen (zoals *B. cinerea*) negatief beïnvloedt, aangezien verhoging van *in planta* ROS gehaltes aanleiding geeft tot een hypersensitieve respons, wat op zich leidt tot verhoogde weefselkolonisatie door necrotrofen. Onze resultaten gaan duidelijk in tegen de algemeen geaccepteerde theorie dat afweer-gerelateerde vorming van ROS de necrotrofe pathogenen helpt in hun pathogeniciteit. Onze resultaten tonen dat een tijdige hyperinductie van waterstofperoxide werkzaam is in de bescherming van de *sitiens* tomatenmutant tegen necrotrofe pathogenen.

Ondanks het feit dat ROS verscheidene functies kunnen hebben in plantafweer, zoals het fungeren als signaalmoleculen, het bezitten van een directe antimicrobiële werking en het leiden tot een hypersensitieve respons, hebben wij aangetoond dat de hyperaccumulatie van waterstofperoxide in *sitiens* leidt tot celwandversteviging, wat resulteert in een inperking van *E. chrysanthemi* en *B. cinerea*. De accumulatie van waterstofperoxide in *sitiens* ging samen met een vroegere en verhoogde activatie van peroxidases en een sterke histochemisch gedetecteerde celwandversteviging op de plaats van pathogeenaanval. Deze bevindingen zijn consistent met de onmiddellijke waterstofperoxide-gevoede peroxidatieve *crosslinking* van stucturele celwandeiwitten en de peroxidatieve inwerking van fenolische moleculen tijdens een *oxidative burst*. Analyse van de genen die in *sitiens* geactiveerd werden tijdens inoculatie met *B. cinerea* toonde tevens aan dat er een verhoogde representatie was van genen betrokken in celwandmodificatie. Microscopische analyse van de vooruitgang van de pathogeen in *sitiens* bladweefsel toonde aan dat zowel *E. chrysanthemi* (op een directe manier gevisualiseerd als intercellulaire micro-kolonies) als *B. cinerea* (op een indirecte manier gevisualiseerd als vooruitgang van de degradatie van pectine) werden tegengehouden door waterstofperoxide-geïnduceerde celwandversteviging. De plaats van de blokkering van de pathogeen (dit is de plaats van celwandversteviging) was verschillend voor *B. cinerea* en *E. chrysanthemi*, wat op

zijn minst gedeeltelijk een gevolg was van de aard van infectieprocedure. *B. cinerea* penetreert de adaxiale cuticula en buitenste periclinale celwand en werd geblokkeerd door verstevigingen ter hoogte van de anticlinale *sitiens* celwanden. De blokkering van *E. chrysanthemi*, dewelke wordt geïnfiltreerd in het bladweefsel, was gelokaliseerd ter hoogte van *sitiens* celwandverstegingen aan de grens van de geïnfiltreerde zone.

Het werd vroeger reeds beschreven dat afzettingen van callose ter hoogte van pathogeenpenetratie beïnvloed worden door ABA. We hebben in dit werk aangetoond dat callose-afzettingen na *B. cinerea* inoculatie zwakker zijn in *sitiens* in vergelijking met wild type. Inhibitie van synthese van callose met 2-deoxy-D-glucose beïnvloedde de resistentie in *sitiens* niet, maar zorgde wel voor een additionele verhoging van ziektegevoeligheid in wild type. Dit toont aan dat callose afzetting geen deel uitmaakt van de *sitiens* afweermechanismen die belangrijk zijn voor resistentie tegen *B. cinerea* en suggereert dat callose afzetting enkel bijdraagt tot basale afweer van wild type tomaat.

Er zijn sterke indicaties dat de snelle en sterke afweer in *sitiens* wordt gelanceerd door herkenning van endogene plant celwandelicitoren. Zowel *B. cinerea* als *E. chrysanthemi* activeren dezelfde afweerreacties in *sitiens*, wat in tegenstelling staat tot de betrokkenheid van pathogeen-specifieke elicitoren. Daarenboven produceren beide pathogenen grote hoeveelheden celwanddegraderende enzymen (CWDEs) en steunen voor een aanzienlijk deel van hun virulentie op de afbraak van pectine. We toonden eveneens aan dat *E. chrysanthemi* mutanten die geen type II secretie vertonen en die dus geen pectinolytische enzymen uitscheiden er niet in slagen de afweer in *sitiens* te activeren. Daarenboven worden deze afweerreacties wel geactiveerd door het cultuurextract van *E. chrysanthemi* met CWDEs en zonder bacteriën. Aangezien we geen verschillen vonden tussen wild type en *sitiens* in de capaciteit om *E. chrysanthemi* celwanddegradatie tegen te gaan, formuleerden we de hypothese dat veranderingen in de celwandsamenstelling van *sitiens* verantwoordelijk zijn voor verschillen in de vrijstelling van celwandoligomeren, welke gekend zijn als krachtige elicitoren van afweerreacties tijdens celwanddegradatie door pathogenen. Anderzijds kan een verhoogde sensitiviteit voor deze oligomeren ervoor zorgen dat *sitiens* zijn afweer sneller kan activeren. We vonden aanwijzingen die beide hypotheses steunen: immunolocalisatie van verschillende celwandcomponenten toonde verschillen aan tussen *sitiens* en wild type in de distributie van pectine en in de aanwezigheid van arabinogalactaneiwitten die waarschijnlijk betrokken zijn bij de signalisatie ter hoogte van de celoppervlakte. Verder onderzoek zal nodig zijn om onze kennis met betrekking tot de activatie van de afweerreacties in *sitiens* te verruimen.

Als conclusie kunnen we stellen dat ABA deficiëntie in tomaat leidt tot *priming* voor pathogeenafweer en dat een snelle hyperinductie van ROS-afhankelijke celwandversteviging een sterke strategie is om de necrotrofe pathogenen *B. cinerea* en *E. chrysanthemi* tegen te houden.

Curriculum Vitae

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2003 Master Degree in Applied Biological Sciences - Cell and Gene Biotechnology - at the Faculty of Bioscience Engineering, Ghent University, Belgium, Distinction; Dissertation: *Identification of AFLP markers linked to resistance to* Magnaporthe grisea *in rice*, Great Distinction

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2005 - 2006 Two-month stage at the Laboratoire Pathologie Végétale (UMR 217 INRA/INA PG/Université P6), Paris, France; Founded by the European exchange program of the Tournesol project T2004.23

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SUPERVISION OF UNDERGRADUATE STUDENTS

2005 - 2006 **Carlos Puig**, Role of abscisic acid and reactive oxygen species in the interaction of tomato with the necrotrophic fungus *Botrytis cinerea*, Promotor: M. Höfte, Thesis to obtain the degree of Bio-engineer, Ghent University

2003 – 2004 **An De Schrijver**, Karakterisatie van Vietnamese isolaten van de rijstpathogeen *Pyricularia grisea*, Promotor: M. Höfte, Thesis to obtain the degree of Bio-engineer, Ghent University